Southeastern Australian agroecosystems:

linking carbon to nutrient dynamics & biotic responses

Thesis for Doctoral candidature

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May 2014

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General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes two original papers published in peer reviewed journals and two unpublished publications. The core theme of the thesis is the role of soil microbial community in decomposition of organic input and elemental cycling. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Faculty of Science under the supervision of Assoc Prof Antonio Patti, Dr Tim Cavagnaro and Dr Cassandra Schefe.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters 1,2,3 and 5, my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
1	Functional stoichiometry of soil microbial communities after amendment with stabilised organic matter	In press	The candidate performed most of the lab work, all the data analysis, and was the primary author of the manuscript.
2	Does the chemical nature of soil carbon drive the structure and functioning of soil microbial communities?	Published	The candidate performed most of the lab work, all the data analysis, and was the primary author of the manuscript.
3	Organic amendment alters resistance and resilience in grassland to drying	Submitted	The candidate ran the experiment, did most of the laboratory work, all the data analysis and was the primary author of the manuscript.
5	Low diffusivity of soil enzymes: an evolutionary and functional perspective.	In prep	The candidate ran most of the lab work, all the data analysis and was the primary author of the manuscript.

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: ……

Date: ……

Summary

Soil is one of the most valuable of common goods. It provides essential ecosystem services including food, nutrient and water cycling, waste treatment, carbon sequestration and greenhouse gas regulation to support plant and human life. However, soils around the world are severely degraded with immense costs to human wellbeing. Addition of organic amendments, in the form of natural organic matter, to soil may allow us to simultaneously improve soil carbon, recycle and replenish nutrients, improve structure, retain water and increase plant productivity. To achieve these improvements, it is necessary to understand the regulation of organic matter transformation. The main aim of this thesis is to examine the chemical and biotic interactions in the transformation of organic inputs in agricultural soils and the impacts of these organic amendments on them. This aim is addressed by examining the interaction between soil carbon, nitrogen and phosphorus cycling and soil microbiota, which play a major role in determining the transformation of organic matter. The findings of the two main experiments are summarised below. The knowledge developed will contribute to managing aagroecosystems for the supply of multiple benefits to the wellbeing of society and agricultural sustainability.

The first experiment focuses on examining the effects of organic input stabilisation via composting or pyrolysis on its transformation by soil biota. The key findings from this experiment are (i) soil microbial composition changes with organic amendment but (ii) overall stoichiometric invariance provides a mechanistic framework for understanding the transformation of organic amendment, and (iii) carbon composition strongly influences microbial community composition and soil functions. Results from this experiment are presented in two chapters.

The second experiment builds on the first experiment by increasing the ecosystem complexity using a terrestrial model ecosystem to better reflect the soil ecosystem in nature. It seeks to determine the interaction between altered rainfall (drying and rewetting) and organic input. In the context of global change, the bigger question asked was, “Does organic input improve the resistance and resilience of a grassland ecosystem to altered rainfall?” The key findings from this experiment are (i) grassland soil microbial communities are generally resistant and resilient to fluctuations in rainfall regardless of compost amendment, and (ii) these properties of the soil microbial community were translated to resilience but not resistance in soil functions, (iii) Plant growth responses were sensitive to rainfall and compost amendment, but interaction effects were rarely detected. Finally, in terms of elemental stock content, (iv) compost amendment increased soil carbon, nitrogen and phosphorus stocks regardless of rainfall regimes but, (v) these subtle improvements can only be detected by careful consideration of depths in the soil profile. Results from this experiment are presented in two chapters. These results supply further weight to the argument that it is necessary to take multiple measures and examine interactions to effectively determine the impact of organic amendments on soil community structure and function, particularly in a world experiencing rapid global change.

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Prologue and acknowledgements: at the beginning

"Begin at the beginning and go on till you come to the end; then stop." Lewis Carroll

I would to start this thesis with some brief comments on my journey as a soil ecologist. This thesis is about the nature of soil. To understand organic input transformation requires an understanding of the nature of soil - its complexity and diversity not only in terms of its biota but also its physicochemical properties. The evolution of the chapters is an indication of this complexity. This is the evidence of the growing pains of the author, whom as she becomes more acquainted with the living invisibles in the soil, hears their whispers of cooperation, competition and relationships of living in communities that reflects that of humanity, albeit at a different scale. But in the end, in an anthropocentric viewpoint, their stories are told to reflect how human influence changes the soil environment and its inhabitants with consequences for human wellbeing.

In May 2011, I arrived at Monash in response to an advertisement for a PhD candidate to undertake novel interdisciplinary studies to understand soil carbon associated with different environments, its turnover and its relationship to soil nutrient cycling. I quickly learnt that one cannot study carbon cycling in a three-year PhD project due to the slow changes in soil carbon pools but it is still possible to understand short-term dynamics and the mechanisms underlying those dynamics. It soon became evident that understanding those short-term carbon dynamics are best understood when coupled with the study of the nutrient cycling and soil biota that are key players to those short-term dynamics. And the plot is never complete without the presence of a villain – a role *Homo sapiens* plays very well. Therefore, we have naturally and extremely heterogeneous soil ecosystems further complicated by

various agricultural interventions. There is always the possibility of reporting observations in the forms of this is what was added to the soil and this is what we observed, which can fill the knowledge gaps very much present in the region. But there is also the question, can we do more? Can we perhaps try to accomplish something that allows the science to move one tiny step closer to being predictive?

As I ventured into the rabbit hole, I wondered: If nature is regulated by some fundamental laws of existence, would we not expect soil to be subjected to these laws? If we can understand the key variables and their interaction, can we better predict the outcomes of human activity? It has been proposed that underlying self-organising nature of any ecosystem is a set of key processes, which interact and feedback, to give rise to the non-additive, emergent property of the ecosystem. In soil, Mark Kibblewhite, Karl Ritz and Michael Swift proposed that carbon and nutrient cycling, together with soil structure maintenance and self-regulation of soil communities are those key variables underlying the self-organisation of soil. The subsequent question then is, which other fundamental laws complement this theory and can further narrow down the properties that need to be examined?

As I ventured deeper into the fundamental laws that govern existence, I stumbled upon ecological stoichiometry. The book “Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere” by Robert W. Sterner and James J. Elser became my bible for the next few months, and I quote them here. “Organisms can be thought of as complex evolved chemical substances that interact with each other and the abiotic world in a way that resembles a complex, composite, chemical reaction. Ecological interactions invariably involve chemical rearrangements. Like any other ‘normal’ chemical rearrangement at the surface of the Earth, when organisms interact, mass must be conserved and elements can neither be created or destroyed. There is stoichiometry in ecology, just as there is in organic synthesis in a test tube. As long as elements are neither destroyed nor created, any

multiple element system will follow stoichiometric rules. However, a driving mechanism behind many of the patterns is the differences in stoichiometric variability among different organisms, species, nutritional modes, trophic levels. The chemical composition of different ecological players is constrained to different degrees, and it is this difference in variation, in addition to differences in mean values, that has interesting effects."

Based on these views of self-organisation and fundamental laws governing existence, Chapter 1 was born. In an incubation experiment, organic amendments of varying stability were employed to examine microbial transformation of organic matter. On one hand, the aim was to understand what regulates soil microbial communities and their transformation of organic inputs. On the other hand, the question asked was can we predict from the knowledge of the regulators what the responses will be? Therefore Chapter 1 asks, "Can stoichiometry provide a mechanistic framework for understanding the transformation of organic inputs to agroecosystems?". In the same incubation experiment, another important regulator of soil microbial community – the chemical nature of carbon in soil and the organic matter – was also examined. To this end, Chapter 2 emerged as another important piece to the story of the transformation of organic inputs to agroecosystems.

While direct human intervention is without a doubt an important driver of change for soil biogeochemical cycling, human-led climate change has led to changes in a very important driver of soil biogeochemical cycling - rainfall. In Chapter 3 and 4, the implications of altered rainfall regime for grassland soil microbial community are examined. Rarely does global change act alone; therefore, it is only realistic to examine the interaction effects of alterations in rainfall regimes and organic inputs. Every ecosystem behaves differently, in part as it retains vestiges of time long gone. It is important to consider an ecosystem response to global change in the context of its past and future potential. The concepts of resistance and resilience allow us to do that, and from this emerges Chapter 3.

Chapter 4 complements Chapter 3, in that it details what happened to the carbon, nitrogen and phosphorus stocks when it rains differently.

Chapter 5 was the result of an endeavour to simplify soil enzyme assay for timely and high throughput examination of soil microbial activity. Although I ultimately failed to create a “kit” as I envisioned, we observed interesting patterns from the trials which led to this chapter on “Low diffusivity of soil enzymes: an evolutionary and functional perspective”.

It was in fact my time at Christian-Albrechts Universität zu Kiel which stimulated my interest in theories. The interesting discussions with Felix Müller, Wilhelm Windhorst and their research team on resilience and ecosystem theories remained deeply entrenched in my mind, albeit my generally poor memory. As I ventured deeper into the rabbit hole and my understanding of all things soil increases, I continued to wonder how ecosystem theories and approaches apply to soil science, some of which I have attempted to apply in this endeavour as described above. As such, I wrote the theoretical Chapter 6 to allow me to explain the unexplained behaviour in soil. It remains to be seen if I should find a way to test those theories or if they can only sustain a hypothetical existence.

This is in the end, as I see it, my last indulgence as a student living only in my head. This is only possible with the support of many others. I would like to express my greatest gratitude to my supervisors, Timothy Cavagnaro, Antonio Patti and Cassandra Schefe who permitted my indulgence. Special thanks to Mick Rose who has been patient with my ideas, entertained them and helped with the pruning to make them more coherent. Kevin Wilkinson, thank you for the many hours of playing around with compost and soil, dealing with my many requests patiently and chats that remind me that we are after all, human. Members of the soil lab and green chemistry lab who assisted me, exchanged ideas or simply stood by and listened to my rambling when all I really needed was an ear after long periods of seclusion.

Tracy Werner, who magically made things appear when necessary. Gentle souls in the labs of Building 17 whom I have relied on for access to this and that equipment, which makes life easier or even possible. Co-authors and colleagues not mentioned above (including and probably not only William Roy Jackson and Wilhelm Windhorst). Monash Sustainability Institute for financial support. Most of all, I thank my family for their patience with me in my endless quest for knowledge, their support and advice when I lose my sight and their belief in me even when I no longer believed in myself.

As with most things in science, there is nothing new under the sun. All of us stand on the shoulders of giants who came before us. It will be impossible to name my many intellectual muses and despite the great injustice that I am probably committing, I shall name the key ones including James J. Elser, Robert W. Sterner, Robert Sinsabaugh, Lance H. Gunderson, C.S. Holling, Patrick M. Lavelle, Petr Baldrian, Richard D. Bardgett, Mark Kibblewhite, Lijbert Brussaard, Michel Loreau, David Wardle and David Tilman.

To find the simple underlying the complex is a creative endeavour. Therefore, besides being a scientific endeavour, each chapter represents a piece of creative endeavour of this author. Each chapter represents a stroke of the brush towards a complete painting. Therefore I hope you enjoy reading it as much as I enjoyed writing it.

"In my hunt for the secret of life, I started my research in histology. Unsatisfied by the information that cellular morphology could give me about life, I turned to physiology. Finding physiology too complex I took up pharmacology. Still finding the situation too complicated, I turned to bacteriology. But bacteria were even too complex, so, I descended to the molecular level, studying chemistry and physical chemistry. After twenty years work, I was led to conclude that to understand life we have to descend to the electronic level, and to the world of wave mechanics. But electrons are just electrons, and have no life at all. Evidently on the way I lost life; it had run out between my fingers." Albert Szent-Gyorgyi

General introduction: organic inputs, soils microbes and elemental cycling

In a time of rising food demand, dwindling fertilizer resources and increasingly degraded soils (Cordell et al., 2009; Lal, 2010), the development of sustainable, biologically regulated nutrient supply soil ecosystems is needed. The addition of organic matter amendments to soil is an option to improve soil nutrients, soil structure and plant productivity (Hargreaves et al., 2008; Quilty and Cattle, 2011). Organic inputs sourced from waste streams such as municipal green waste have the added benefit of diverting large quantities of waste from landfills. For example, that would mean reducing 36% of solid waste going to landfill in Victoria, Australia in 2002 (Nolan-ITU, 2002). More recently, incorporation of organic amendments in stabilised form has also been proposed to benefit long-term carbon sequestration (Lal, 2011; Lehmann, 2007).

Organic inputs can contain substantial amounts of nutrients but the processes leading to their decomposition and persistence as soil organic matter remains unclear. It is likely that decomposition of organic inputs depends on the interplay of physical protection or accessibility to microbes, chemical stability[1] and stabilisation by organo-mineral interactions (Ekschmitt et al., 2005; Kögel-Knabner et al., 2008). These controls on decomposition are in turn affected by human activity, parent material, below-ground and above-ground biota and the climate (Schmidt et al., 2011). To add to the complexity, the relative importance of the controlling factors is likely to change over time and spatial scale. There are also abiotic processes independent of biota leading to decomposition and stabilisation of organic matter

[1] Some would call it inherent recalcitrance, but this definition is disputable and it is increasingly questioned if there is really something that the microbes cannot digest. This question deserves an entire dissertation by itself and will not be further discussed here. Chemical stability is defined here as in terms of molecular properties including high proportion of aromatics and low aqueous solubility.

(Kemmitt et al., 2008; Wallis et al., 2011). While these abiotic processes are by no means unimportant, this thesis focuses on the living biota, which albeit being small in biomass relative to the total soil biomass are big in terms of effects on ecosystem behaviour and processes (Bardgett and Wardle, 2010; Conrad, 1996; Mulder et al., 2011; Philippot et al., 2013). Furthermore, soil biota, whose fundamental nature is to survive and reproduce provides us an opportunity to encourage them in the direction we find meaningful with the right knowledge and tools. The addition of organic matter amendments to soil is one such attempt.

Soil microbial communities play a critical role in the decomposition and transformation of soil organic matter (Baumann et al., 2012; Dungait et al., 2012; Ekschmitt et al., 2005). Microbial activities regulate several greenhouse gas fluxes at a global scale (Conrad 1996) including CO_2, N_2O and CH_4. Besides participating in biogeochemical cycling, soil microbes represent an important pool of labile nutrient in soil. On a global scale, total soil bacteria are estimated to contain 26pg of carbon (4-5% of total carbon in plants), 6.2pg N and 0.65pg P (almost equal to the amount of these nutrients in terrestrial plants) (Whitman et al., 1998). Soil stores more carbon than plants or the atmosphere on a global scale, and therefore, contributes significantly to carbon cycling and feedbacks to climate (Kleber, 2010). Although we know the importance of soil biota and their processes for global biogeochemical cycling, we know little of the regulation of the soil microbial community and how the regulatory variables vary within a backdrop of soil's complex physico-chemical environment.

The response of the soil microbial community to organic inputs depends in part on the stoichiometric constraint of elemental composition of their biomass (Cleveland and Liptzin, 2007; Sterner and Elser, 2002). In a carbon-limited system, microbial response to nitrogen and phosphorus input differs from that of a carbon-unlimited system and vice versa (Craine et al., 2007; Knicker, 2011; Schimel and Weintraub, 2003). This stoichiometric constraint of

elemental composition observed in microbes is also observed higher up in the food web. As such, there is considerable range (in terms of time and space) of responses that may be expected with short term change in resource quality (Sistla and Schimel, 2012; Sterner and Elser, 2002). This means that changes in resource quality, such a result of organic input, may influence macro-scale processes such as that of carbon, nitrogen and phosphorus cycling. Such interactions indicate the importance of examining carbon, nitrogen and phosphorus dynamics concurrently to understand organic input transformations and their implications for biogeochemical cycling.

The response of the soil microbial community to organic inputs is also strongly influenced by the carbon composition of the organic input and the soil ecosystem (Fontaine et al., 2007; Grandy and Neff, 2008; Kramer and Gleixner, 2006). The elemental and molecular composition of the organic input affects its residence time as it influences biological stabilisation, physical protection and the energy required for its breakdown (Ekschmitt et al., 2005; King, 2011; Kögel-Knabner et al., 2008; Schmidt et al., 2011). For example, as organic matter decomposition increases, less stable structures are degraded and the relative proportion of aromatic and aliphatic structures in soil organic matter increases (Gressel et al., 1996; Pedersen et al., 2011). Phenolic compounds have been correlated with the antioxidant capacity of soils that neutralise free radicals, and therefore, protect organic matter from oxidation (Rimmer and Abbott, 2011). Aromatic compounds have also been implicated in hydrophobic protection of soil organic matter (Spaccini and Picollo, 2012). Aliphatic and other organic carbon compounds have been found to associate with soil humic material to form supramolecular structures, thereby offering them hydrophobic protection (Piccolo, 2002; Sutton and Sposito, 2005).

Given the chemical complexity of soil organic matter, a wide range of soil enzymes are involved in organic matter transformations (Allison and Vitousek, 2004; Sinsabaugh,

2010). For example, hydrolytic enzymes, such as β-glucosidase and phosphatases, catalyse specific steps in mineralisation of carbon and phosphorus (Shi, 2010), whereas oxidative enzymes, such as phenol oxidases, act more generally on phenolic-containing compounds including lignin, humus and polyphenols (Sinsabaugh, 2010). Enzyme encounter with substrate occurs largely by diffusion (Koch, 1990) and enzyme production is costly to microbes. As such, soil microbial activity reflects not only the substrate supply but also favourable local conditions and preferential substrate (Ekschmitt et al., 2005). It has been suggested that quality above quantity of organic matter controls extracellular enzyme activity (Moorhead et al., 2012). It has been observed that the chemistry of decomposing plant litter exposed to different decomposers becomes more dissimilar rather than similar during decomposition (Wickings et al., 2012). Distinct soil microbial communities typically have dissimilar metabolic traits due to different carbon preferences of the microbial groups (Kramer and Gleixner, 2008; Wickings et al., 2012). For example, Gram positive bacteria have been found to preferentially use carbon derived from soil organic matter, whereas Gram negative bacteria preferentially use carbon derived from plant derived inputs such as litter, roots and root exudates (Kramer and Gleixner, 2008). It is also proposed that fungi dominate the decomposition of carbon-rich and nutrient-poor organic matter such as lignin, whereas bacteria dominate the decomposition of nutrient-rich organic matter such as proteins (Keiblinger et al., 2010; Van Der Wal et al., 2006; Waring et al., 2013). Furthermore, the degradation of lignocellulose rich organic matter by oxidative enzymes is dominated by fungi (De Boer et al., 2005; Kirk and Farrell, 1987; Van der Wal et al., 2013). Through a combination of competitive exclusion and coexistence mechanisms, soil microbial community adjusts to variations in resource stoichiometry[2] (Danger et al., 2008; Fontaine et al., 2003; Moorhead and Sinsabaugh, 2006). The fungal-based food web has been linked to

[2] Resource stoichiometry here refers to ratios of the elements, i.e. the C:N:P ratios. Resource for the soil microbes can be the organic inputs, litter materials or soil organic matter.

slower decomposition and therefore slower nutrient cycling while bacteria-based food web is characterised by faster decomposition and nutrient cycling (Moore et al., 2003; Wardle et al., 2004). This has important implications for the choice of organic amendments for sustainable nutrient recycling in ecosystems.

The feedback between soil nutrient cycling and plants also affects long-term fertility and productivity of the ecosystem (Wardle et al., 2004). Given the importance of plant-soil feedbacks, a study of soil responses to organic inputs is incomplete without examining the interaction between the soil microbial community and plants. Soil microbes and plants are closely tied by a biological market place, where microbes in nutrient cycling, organic matter turnover and decomposition secure nutrient supply to and from plants to maintain the food web. In a study on nitrogen-limited soils by Zak et al. (2003), greater plant productivity was associated with greater microbial biomass, respiration, fungal abundance and nitrogen mineralisation rate. In some cases, trading partners establish tight relationships such as that seen in the symbiotic relationship between plants and mycorrhizas (Kiers et al. 2011). These feedbacks between soil organisms and plant community have important implications on soil processes (Bardgett and Wardle 2010). In a seven year field manipulation experiment, it was observed that as plant diversity increases, microbial biomass and nitrogen cycling rate also increased (Zak et al., 2003). In another study, Kuzyakov and Xu (2013) proposed that temporal niche differentiation between plant roots and microbes protects ecosystems from nitrogen losses by leaching. Plants traits also regulate net soil carbon storage by affecting carbon assimilation through decomposers, its release from soil through respiration, fire or leaching, or by altering soil abiotic conditions (De Deyn et al., 2008).

Finally, human activities have caused rapid change in the climate and these have important implications of the transformation of organic matter and biogeochemical cycling. In the Mediterranean-like climate of much of southeastern Australia, soil organisms are likely

to have adopted a range of lifestyles to survive climatic instability, particularly in relation to water availability (Griffiths et al., 2003; Waldrop and Firestone, 2006). Despite the fact that ecosystems everywhere are subjected to multiple antropogenically driven environmental changes, our understanding of ecosystem responses to simultaneous global change drivers remains incomplete (Dukes et al., 2005). There is a particularly pressing need for research on below-ground responses to multiple global environmental change factors acting in concert (Eisenhauer et al., 2012).

As climate projections suggest a future in southeastern Australia with an increase in the frequency and severity of drought and extreme rainfall events (Alexander and Arblaster, 2009; Hennessy et al., 2008), how soil microbial communities will withstand or adapt to these changes remains unknown. There are few studies that have addressed this question at the ecosystem scale (Chou et al., 2008; Dukes et al., 2005; Weltzin et al., 2003) and studies looking at water stress are useful starting points (e.g. Borken and Matzner, 2009; Clark et al., 2009; Fierer et al., 2003; Unger et al., 2009; Van Gestel et al., 1993). These studies suggest that alterations in rainfall regimes are likely to affect soil microbial community directly through moisture, temperature and nutrient availability and indirectly through the plant feedback and changes in soil structure, soil pH and soil surface hydrophobicity. The differences in microbial responses to the alteration in rainfall regimes may affect soil microbial activity and alter the dynamics of ecosystem processes. In combination with other drivers of global change, the responses of an ecosystem can be highly variable. As such, examining these responses in the context of resistance and resilience allow us to connect the present conditions to soil historical legacy and its future potential.

Currently, agriculture dominates 488,369,772 km^2 or approximately 38% of global land area (FAOSTAT, 2010). As crop yield, soil health and climate are tightly linked (Lal, 2010), the right intervention recipe will allow the restoration of soil organic matter and

benefit food security, poverty alleviation and mitigate climate change. Understanding soil microbial transformation of soil organic matter and the implications of climate change on their behavior and responses as a community is a step towards achieving that. Towards this aim, this thesis investigates the following research questions.

1. The incorporation of organic inputs in stabilised form is proposed to benefit nutrient supply and improve long-term soil carbon content. The effects on, and the responses of, soil microbial communities to such organic inputs are unclear. In an incubation experiment using two contrasting agricultural soils, the effects of a green waste and its compost or biochar are examined. The key relationships between soil microbial community composition, biomass and enzyme activity, with resource elemental composition, carbon composition and nutrient availability are examined. Information from ^{13}C-NMR is used to determine the effects of carbon composition on soil microbial community. An attempt is also made to identify the mechanisms underlying responses observed using a combination of ecological and functional stoichiometry theories (Sinsabaugh et al., 2009; Sinsabaugh et al., 2008; Sterner and Elser, 2002). This work has been published in Soil Biology and Biochemistry (Ng et al., in press; Ng et al., 2014).

2. Currently, the ability of the soil microbial communities in this region to cope or adapt to variability in rainfall is unknown. Organic inputs have been suggested to improve soil resilience to disturbance (Griffiths and Philippot, 2013). For example, organic matter amended soils have been observed to exhibit less pronounced changes in PLFA compared to unamended soils under drought conditions (Hueso et al., 2012). A second major experiment followed, whereby, information from the incubation experiment is used to select organic input that elicits the most positive responses from soil microbial community, with consideration of longevity of these responses. This second experiment involved increasing

the ecosystem complexity using a terrestrial model ecosystem and taking into consideration climatic variable. In the context of global change, the bigger question asked is, “Does organic input improve the resistance and resilience of a grassland ecosystem to altered rainfall?”. This work has been submitted for publication (Ng et al., submitted).

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Declaration for Thesis Chapter 1

Declaration by candidate

In the case of Chapter 1, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Setup study design, performed most of the lab work, analysed all the data and wrote the manuscript.	90

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Antonio Patti	Involved in study design, writing manuscript	2
Timothy Cavagnaro	Involved in study design, writing manuscript	2
Michael Rose	Microbial biomass analysis, writing manuscript	2
Kevin Wilkinson	Involved in study design, prepared organic amendments, conducted fieldwork, writing manuscript	2
Cassandra Schefe	Involved in study design, prepared organic amendments, conducted fieldwork, writing manuscript	2

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Chapter 1: Functional stoichiometry of soil microbial communities after amendment with stabilised organic matter

This chapter has been accepted for publication in Soil Biology and Biochemistry.

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Abstract

The transformation of organic matter amendments in the soil is regulated by soil microbial communities. We examined the utility of ecological and functional stoichiometry theories to explain microbial transformation of organic amendments in the soil and examined the key relationships between soil microbial community composition, biomass and activity with resource elemental composition (soil and organic input) and nutrient availability. Using two contrasting soils amended with raw green waste, its compost or biochar, we found that microbial PLFA composition was distinct for each soil and organic amendment. Microbial activity was strongly influenced by organic amendment. Further, we observed that changes in the soil stoichiometry with inputs were accompanied by changes in total PLFA and

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bacteria:fungal ratio, but the relationships between them were inconsistent and changed over time. Microbial activities involved in C, N and P cycling were generally correlated, but the relationship between hydrolase β-glucosidase (BGL) and microbial N and P activities was stronger and more consistent than that between oxidases (phenol oxidase PPO, peroxidase POX) and microbial N and P activities. These microbial activity relationships translated to a consistent relationship between log(BGL):log(nutrient) and soil C:nutrient but a weaker and inconsistent relationship between log(PPO+POX):log(nutrient) and soil C:nutrient. Our analyses indicate that microbial composition can be different, but stoichiometric invariance of microbial activity constrained microbial community response to organic input.

Keywords: soil enzymes; nutrient cycling; soil microbial community; ecological stoichiometry; functional stoichiometry

1.1. Introduction

In a time of dwindling global fertilizer resources (Cordell et al., 2009) and rising food demand, there is growing interest in the development of an agricultural paradigm based around sustainable biologically regulated nutrient supply systems. The addition of organic amendments, such as nutrient rich plant residues and composts, to soils can deliver nutrients and organic matter that improve soil structure and plant productivity (Hargreaves et al., 2008). In Victoria, Australia, municipal waste comprises approximately 1.6 million tonnes or 36 % of solid waste going to landfill in 2002 (Nolan-ITU, 2002). The use of such waste as organic amendment allows large quantities of waste to be diverted from landfills. In recent years, incorporation of such organic amendment in its stabilised form is emerging as management option due to its added potential for long-term carbon sequestration (Lal, 2011; Lehmann, 2007). If widespread use of organic amendments to supply nutrients for plant

productivity is to become a viable option to offset the use of inorganic fertilisers in agriculture, and potentially improve soil carbon content, their use must be predicated upon a complete understanding of the soil ecological processes that govern their fate in soil (Jackson et al., 2008).

Soils contain arguably the most diverse terrestrial communities on the planet (Wardle, 2006), and vary considerably on scales ranging from the microsite (α-diversity) to landscape (γ-diversity) (Ettema and Wardle, 2002). While the importance of soil microbial communities in organic matter decomposition is well recognised, the factors that regulate microbial-mediated carbon transformation and nutrient release are still being debated (e.g. Dungait et al., 2012; Schmidt et al., 2011). If we are to discover the factors regulating the fate of organic amendment in the soil, a theoretical framework that effectively deals with the tremendous structural and functional complexity in soils must guide our work.

Ecological stoichiometry, that is, the balance of chemical elements and the concept of inflexible elemental ratios (particularly of C, N and P), has been proposed to explain ecosystem processes (Sterner and Elser, 2002). In a study on plant litter – microbe system, C:nutrient ratios of the soluble litter fraction have been linked to shifts in microbial community composition (Fanin et al., 2013). The ratios of C:N:organic P:S in the humus fraction of soil organic matter was reported to be constant for a wide range of soils from Australia and elsewhere under different land uses (Kirkby et al., 2011). These results suggest that while microbial community composition varies from ecosystem to ecosystem, and in response to organic amendment addition, the overall stoichiometry of soil organic matter proceeds toward a constant equilibrium ratio.

Soil microbial communities are regulated by both the supply of energy and nutrients (especially N and P) (Hessen et al., 2004). In Ng et al. (2014; see Chapter 2), we found that

carbon composition of the organic input explained at least 50% of the variation in microbial community composition and activity in soils treated with different organic inputs. While previous studies have shown that the chemical nature of the organic amendment affects microbial community composition and activity; few studies have specifically examined the underlying mechanisms that regulate these responses (Bowles et al., 2014; Fanin et al., 2013; Güsewell and Gessner, 2009; Kallenbach and Grandy, 2011). Microbial demand and use of resources is driven by the elemental stoichiometry of their biomass, but is regulated by the elemental stoichiometry of the resources (Cleveland and Liptzin, 2007; Sterner and Elser, 2002). The balance of these competing constraints is hypothesised to be captured by the functional stoichiometry of the microbial community (Sinsabaugh et al., 2009); that is, the ratio of the activity of microbial processes involved in the cycling of C, N and P (i.e. C:N:P activity ratios). Specific ecoenzyme activities (e.g. β-glucosidase, amidase, peroxidase, and phosphatase) provides a reliable measure of functional stoichiometry which integrates the stoichiometric and metabolic theories of ecology, and links microbial metabolic efficiency to microbial biomass and the elemental composition of their food resources (Allen and Gillooly, 2009; Sinsabaugh et al., 2009; Sinsabaugh et al., 2008). Past studies have generally examined functional stoichiometry at landscape and global scales (Sinsabaugh et al., 2009; Sinsabaugh et al., 2008; Sinsabaugh and Shah, 2011). These studies have identified a constrained functional stoichiometry in response to climatic and edaphic variables. While studies have shown broad global patterns in functional stoichiometry, the relevance of this theoretical framework to farm scale variations due to differences in management inputs has not yet been explored.

In this study, we explore the utility of ecological and functional stoichiometry theories to understand the microbial transformation of three organic amendments differing in elemental stoichiometry as a result of composting and pyrolysis processes, upon addition to

soil. Our goal is to examine the key relationships between soil microbial community composition, biomass and activity, with resource elemental composition and nutrient availability. To do so, we examined the effects of green waste (raw, composted or pyrolysed) addition on nutrient availability and soil microbial community composition and activity in two contrasting soils. We measured the activity of two oxidases (peroxidase and phenol oxidase) that are known for their role in stable organic matter breakdown, and the more commonly measured hydrolase, β-glucosidase. We also measured potentially mineralisable nitrogen and alkaline phosphatase activity as proxies for/indicators of N and P cycling. We hypothesised that the functional stoichiometry of the soil microbial community would correlate well with input stoichiometry initially, and as the added organic amendments decomposed, the functional stoichiometry would evolve to better reflect the overall soil stoichiometry. We expect this relationship to be consistent across ecosystems, i.e. the distinct soil microbial communities in both soils experience similar stoichiometric constraints.

1.2. Materials and methods

Experimental set-up

This paper is the second arising from an experiment in which we assessed the fate of green waste-derived OA in soils. Whereas the first paper dealt with the fate and cycling of C present in the soil in different forms (Ng et al., 2014; see Chapter 2), this paper focuses on soil N and P cycling. A microcosm-based incubation study was conducted. Organic matter derived from municipal green waste was added to two soils, in either its raw state, or following composting or pyrolysis of the green waste (see Ng et al. (2014) on preparation of the organic amendments).

The Cranbourne soil, which was collected from a horticultural farm in Cranbourne, Australia (38°11' S 149°19' E), was a semiaquic Podosol, loamy sand, pH 7.79 (H_2O); mass ratio C:N:P (73.8:10.5:1); organic matter (1.3 %); water holding capacity (20.8 %). The Werribee soil, which was collected from a horticultural farm in Werribee, Australia (37°53' S, 144°40' E), was a strongly dispersive (basaltic) red Sodosol (Isbell 1996), slightly sodic light clay topsoil, pH 7.79 (H_2O), mass ratio C:N:P (26.5:2.5:1); organic matter (3.9 %); water holding capacity (49.5 %). Both soils were collected from the top 10 cm soil layer, air dried and sieved to < 2 mm. The organic amendments added to the soils were a raw green waste or its composted or pyrolysed forms, which are referred to as green waste, compost and biochar, hereafter (see supplementary table S1.1 for properties). For details on the composting and pyrolysis, see Ng *et al.* (2014; see Chapter 2). Before they were applied, the green waste and compost were sieved to 12.5 mm. The green waste was additionally passed through a garden mulcher before sieving. The biochar did not require further pre-processing prior to application due to its small particle size. The organic amendments were added to 300 g of either soil; these application rates correspond to an increase in total soil C of ca. 1%. Unamended controls were also included. The soils were wet up to between – 30 and – 40 kPa and incubated at 25 °C. Soil moisture content was maintained during incubation by the addition of water after weighing the soils every 4 to 7 days. Each treatment was replicated four times and sampled at 4 weeks and 12 weeks. Samples were air dried for chemical analysis, kept at 4 °C for enzyme analysis or -20 °C for all other analyses.

Microbial analysis

Soil microbial community structure was assessed by phospholipid fatty acid (PLFA) analysis. PLFA was extracted using a method modified from Bligh and Dyer (1959) with

addition of citrate buffer (Nielsen & Petersen 2000) followed by alkaline methanolysis of phospholipids (Bossio and Scow, 1998). PLFA determination was done using a Varian CP 38/00 gas chromatograph fitted with 5 % phenyl:95 % methylsiloxane column (Varian, Walnut Creek CA, USA). The fatty acids i15:0, a15:0, 15:0, i16:0, 16:1ω7, i17:0, a17:0, 17:0cy, 17:0, and 19:0cy were chosen as bacterial biomarkers and linoleic acid (18:2ω6,9) was chosen as the biomarker for decomposer fungi (see Frostegård and Bååth, 1996 and references therein). We did not include 18:1ω9 as a fungal marker in our analysis, as this marker is also found in plants and bacteria and it is a poor indicator of fungi in agricultural soils (Frostegård et al., 2011). Concentrations of fatty acids less than 0.1 ppm were treated as 0 and only fatty acids detected in > 4% of treatments were included in the analysis which included 21 PLFAs. Total PLFA was used as a relative measure of soil microbial biomass (Frostegård and Bååth, 1996).

β-glucosidase (BGL) and phosphatase (PHOS) are hydrolases involved in cellulose degradation and mineralisation of organic phosphorus respectively (Shi, 2010). Phenol oxidase (POX) and peroxidase (PPO) are oxidases associated with the degradation of recalcitrant carbon forms (Sinsabaugh, 2010). The activities of BGL, PHOS and PPO were determined according to methods modified from Allison and Jastrow (2006). POX was determined according to Frey et al. (2000) and Johnsen and Jacobsen (2008). Briefly, we incubated 0.5 ml of soil slurry (1 g soil in 50 ml sterile H_2O) with 0.5 ml of substrate solution for 2 hr (BGL and PHOS), 1 hr (PPO) or 10 mins (POX). We used a buffer of pH 7 (3-(N-morpholino)propanesulfonic acid or MOPS, 100 mM) where possible to match our soil pH, except for POX assay for which acetate buffer (pH 5, 100 mM) was used to make up the substrate solution. *p*NP-β-D-glucopyranoside (5 mM), *p*NP – phosphate (5 mM), pyrogallol/EDTA (50 mM/ 50 mM) and 3,3',5,5'- tetramethylbenzidine (TMB; 1 mM) were substrates for BGL, PHOS, PPO and POX respectively. A background soil control and a

substrate control were prepared for all enzymes. Colorimetric measurement of reaction products was made at absorbance 405 nm except for POX which was measured at 450 nm. The PPO standard was obtained by measuring the absorbance of completely oxidised products of known amounts of pyrogallol with commercial mushroom tyroxinase (1mg/ mL). A standard curve of absorbance versus *p*-nitrophenol was used for BGL and PHOS. The extinction coefficient was obtained from the manufacturer for TMB. Changes in N activity were measured using potentially mineralisable nitrogen (PMN) as a proxy measure in 5 g soil using an anaerobic incubation at 37 °C for 7 days as described by Waring and Bremner (1964). The ammonium (NH_4^+) was extracted with 4 M KCl and measured colorimetrically following Forster (1995) modified for 96-well microplate. Two reagent blanks and immediate NH_4^+ extracts were made using 2 M KCl.

Chemical analysis of soils and organic amendments

The organic amendment, unamended and amended soil samples were also analysed for a suite of chemical properties. Mineral N (NO_3, NH_4) and $CaCl_2$-P (which is a measure of labile P fraction) were determined as described in methods 7C2, and 9F1 of Rayment and Higginson (1992). A high-frequency induction furnace (LECO Pty Ltd) was used to measure total soil C and N. Total soil P was determined by perchloric acid digestion before analysis by inductively coupled plasma atomic emission spectroscopy (Spectro Analytical Instruments Pty Ltd, Kleve, Germany).

Statistical analysis

C:nutrient stoichiometry was calculated using %N, %P and %C of soil dry weight (i.e mass ratio). Input stoichiometry is determined from the organic amendment at the start of the experiment. Non-metric multidimensional scaling (NMDS), an unconstrained ordination analysis (Legendre and Legendre, 2012), was performed to assess general trends in microbial community composition and soil function with organic amendment and over time. Redundancy analysis (RDA), a constrained ordination analysis (Legendre and Legendre, 2012), was used to identify the environmental variables that explain the trends in microbial PLFA composition and activity (i.e. PHOS, PMN, BGL, PPO and POX). The ordination plots display similar objects close to one another while dissimilar objects are farther apart. For multivariate analysis, all data were standardized accordingly as follows: PLFA data was log (n+1) transformed and standardised by chord transformation, microbial activity was standardised by chord transformation and environmental variables (i.e. soil NH_4^+, soil NO_3^-, soil C:N ratio, soil C:P ratio, initial C content upon addition of organic amendment or of unamended soil, in the case of control) were standardised to zero mean and unit variance. For the NMDS, Bray-Curtis distance matrix was calculated using the above standardised data and NMDS performed using metaMDS() function in *vegan* package. The general trends observed in NMDS agree with patterns observed in the RDA. NMDS is presented in the main text and the RDA is provided in the supplements. NMDS and RDA were plotted as 95 % confidence ellipses using the component scores for each of the four replicates.

Kendall τ is a nonparametric rank correlation method to quantify the relationship between two variables (Legendre and Legendre, 2012). Kendall τ correlations were calculated using log (n+1) transformed data for the amended and unamended soils' C:nutrient ratios at sampling, input (organic amendment) C:nutrient ratios and functional C:nutrient

ratios. In the calculation of average ratios for functional stoichiometry, unrepresentable (NaN) and infinite (Inf) values that appeared at 12 weeks due to undetectable activity level were excluded. Two-way analysis of variance (ANOVA) was carried out for total PLFA and bacterial: fungal ratio to examine the effect of organic amendment and soil. Data was checked for homogeneity of variance and normality. Where the assumptions were violated, data was transformed and compared to the untransformed data. As the results were similar with both transformed and untransformed data, we have retained all analysis using the untransformed data. Where significant interaction between soil and organic amendment were found, we compared differences between organic amendments within each soil using least significant difference test with *p*-values adjusted using Bonferroni.

Data analysis was carried out on R 2.15.1 (R Core Team 2012) using *vegan* package (Oksanen et al., 2012) for ordinations and *agricolae* package (Mendiburu, 2012) for Kendall correlations.

1.3. Results

Stoichiometry of the resources

The input C:N ratio was highest in biochar (20.9) followed by green waste (14.2) and compost (10.7). The input C:P ratio was highest in green waste (88.5) followed by biochar (63.3) and compost (53.6). The initial C:N ratios of the Cranbourne and Werribee soils were 7.0 and 10.6 respectively; and the initial C:P ratios of the Cranbourne and Werribee soils were 73.8 and 26.5. The total soil C, N and P were positively correlated at 4 and 12 weeks (Table 1.1A, Fig. 1.1). Patterns in the total soil C, N and P were similar in both soils, i.e. compost amended soils had the highest total C, total N and total P at 4 and 12 weeks. Green

waste and biochar amended soils also had higher total C, total N and total P than unamended soils at both time points. Soil stoichiometry was inconsistently correlated with input stoichiometry (Table 1.1B). There was a positive correlation between soil C:N with the input C:N ratio only at 12 weeks and soil C:P ratio with input C:P ratio only at 4 weeks.

Table 1.1. Kendall τ correlation of (A) soil C, N and P totals; (B) ratios of OA input C:nutrients, soil C:nutrient and C:nutrient activity; (C) ratios of bacteria:fungal ratio and total PLFA with ratios of OA input C:nutrient and soil C:nutrient; (D) microbial C activity (BGL or PPO+POX) in relation with microbial N activity (PMN) and microbial P activity (PHOS). Significant levels are *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

	Week 4		Week 12	
A	total soil C	total soil P	total soil C	total soil P
total soil N	**0.85 *** **	**0.85 *** **	**0.81 *** **	**0.8 *** **
total soil P	**0.87 *** **		**0.90 *** **	
B	input C:N	soil C:N	input C:N	soil C:N
soil C:N	0.23		**0.38 ****	
BGL/ PMN	-0.18	**-0.61 *** **	-0.21	**-0.52 *** **
(PPO+POX)/ PMN	**- 0.30 **	0.15	-0.06	**0.30 **
	input C:P	soil C:P	input C:P	soil C:P
soil C:P	**0.49 *** **		0.26	
BGL/ PHOS	-0.04	**-0.41 ****	-0.06	**-0.49 ****
(PPO+POX)/ PHOS	-0.02	**0.34 ****	-0.05	0.27
C	bacteria:fungal	total PLFA	bacteria:fungal	total PLFA
soil C:N	-0.05	**-0.31 **	**-0.30 **	**-0.61 *** **
soil C:P	0.01	-0.17	-0.16	**-0.37 ****
input C:N	**0.32 **	0.05	0.25	-0.10
input C:P	**0.32 **	0.11	0.25	-0.14
Soil NH_4^+	**0.32 **	**0.61 *** **	**0.47 *** **	**0.52 *** **
Soil NO_3^-	0.01	0.23	0.22	**0.52 *** **
D	PMN	PHOS	PMN	PHOS
BGL	**0.42 ****	**0.68 *** **	**0.40 ****	**0.61 *** **
PPO+POX	-0.08	-0.04	**0.24 **	**0.48 *** **
PHOS	**0.41 ****		**0.59 *** **	

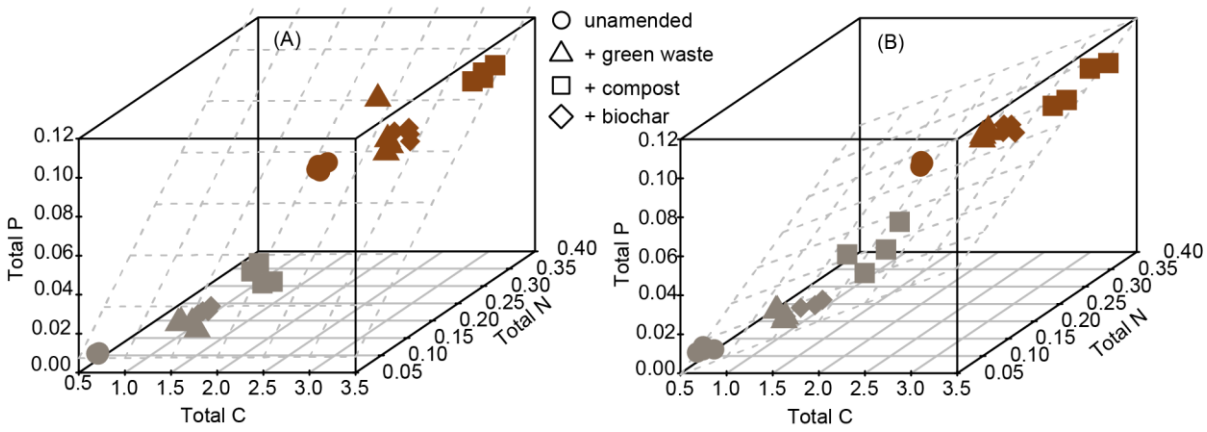

Fig. 1.1 Total soil C in relation with total soil N and total soil P for 4 (A) and 12 (B) weeks. Brown = Werribee soil, grey = Cranbourne soil. Units are in g/ 100g. Regression plane serves only for illustration of the relationship among the C,N and P. Correlation between total soil C, total soil N and total soil P analysed using Kendall τ (table 1.1B).

Soil microbial community composition, size and structural responses to amendments

Soil microbial biomass, measured as total PLFA, differed markedly among the soils and organic amendment at both time points (Fig 1.2A, 1.2C, see supplements for ANOVA table). The Werribee soil generally had higher microbial biomass than the Cranbourne soil, irrespective of sampling time or organic amendment. Whereas the addition of biochar to the soils resulted in similar or even lower total PLFA than their respective unamended soils, the addition of green waste and compost resulted in significant increases in total PLFA, particularly at 4 weeks.

The bacterial:fungal ratio also changed with organic amendment. For each soil, the bacterial:fungal ratio was consistently highest in unamended soils, and lowest in green waste amended soils, at both time points (Figure 1.2B, 1.2D). Compost and biochar amended Werribee soil had similar bacterial:fungal ratios, whereas the biochar amended Cranbourne

soil had higher bacterial:fungal ratio than the compost amended Cranbourne soil at both time points.

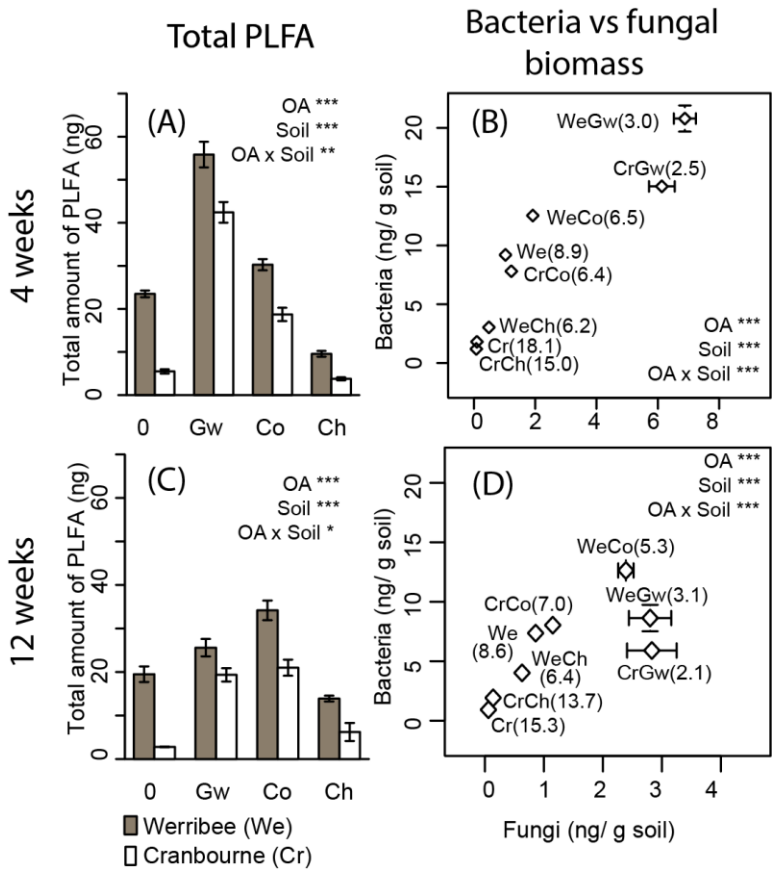

Fig. 1.2(A) Total PLFA and (B) bacteria to fungal biomass (mean ± se) for 4 weeks. (C) Total PLFA and (D) bacteria to fungal biomass (mean ± se) for 12 weeks. Numbers in bracket refer to bacteria:fungal ratio for each treatment in (B) and (D). . Significant levels are *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (refer to supplementary table S3 for test statistics).See table 1.1C for correlation between bacteria:fungal ratio and total PLFA with edaphic properties. Samples refer to We = Werribee unamended soil, Cr = Cranbourne unamended soil, WeGw = Werribee soil + green waste, CrGw = Cranbourne soil + green waste, WeCo = Werribee soil + compost, CrCo = Cranbourne soil + compost, WeCh = Werribee soil + biochar, CrCh = Cranbourne soil + biochar.

Based on NMDS, the variation between microbial compositions in the Cranbourne soil with different amendments was greater than the variation between microbial compositions among Werribee soils with different organic amendment at 4 weeks (Fig 1.3A).

The non-overlapping confidence ellipses in the ordination plot indicated that the microbial composition was unique with each treatment. At 12 weeks, microbial composition in compost and biochar amended Werribee soils diverged from unamended and green waste amended Werribee soils. In contrast, in Cranbourne soils, the microbial composition of biochar amended soil was more similar to the unamended soil and compost amended soil remained least similar to other treatments.

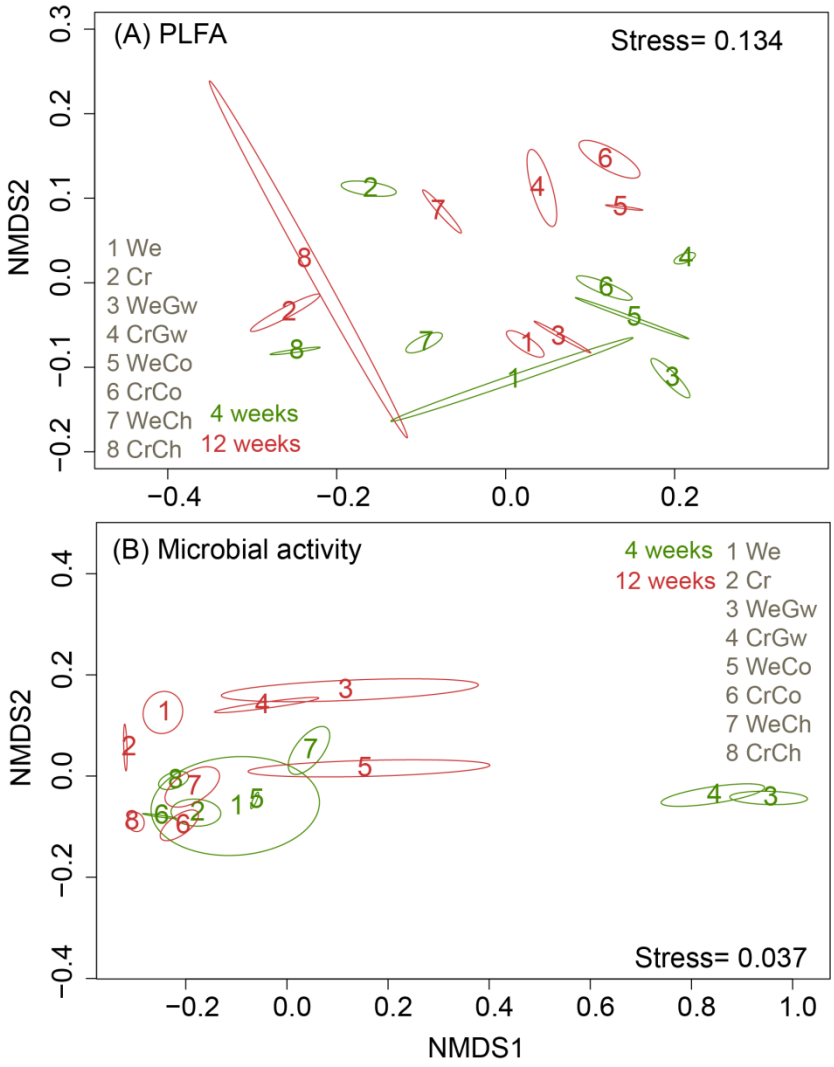

Fig. 1.3 NMDS ordination plot based on (A) microbial PLFA composition and (B) microbial activity. Samples refer to 1 = We, Werribee unamended soil, 2 = Cr, Cranbourne unamended soil, 3 = WeGw, Werribee soil + green waste, 4 = CrGw, Cranbourne soil + green waste, 5 = WeCo, Werribee soil + compost, 6 = CrCo, Cranbourne soil + compost, 7 = WeCh, Werribee soil + biochar, 8 = CrCh, Cranbourne soil + biochar.

Relationships between microbial structure, resource stoichiometry and mineral nutrients

Total PLFA was negatively correlated with soil C:N ratio at both 4 and 12 weeks, and negatively correlated with soil C:P ratio only at 12 weeks (Table 1.1C). Total PLFA was not correlated with input C:nutrient at any time point. Total PLFA was also positively correlated with soil NH_4^+ at both time points but the relationship with NO_3^- was inconsistent (Table 1.1C). The bacterial:fungal ratio was negatively correlated to soil C:N ratio at 12 weeks and positively correlated to input C:nutrient ratios at 4 weeks (Table 1.1C). The bacterial:fungal ratio was positively correlated with NH_4^+ at both time points but showed no relationship with NO_3^- (Table 1.1C).

The shifts in the soil microbial composition with addition of organic input, at both time points, were partly explained by soil type, soil C:N ratio, soil C:P ratio, initial C content, soil NH_4^+ and soil NO_3^- (Supplementary Fig. 1.1A). Based on RDA, 46.7% of total variance in the microbial composition was accounted for by these variables.

Soil microbial activity responses to amendments

The microbial C, N and P activities were generally positively correlated at 4 and 12 weeks except for the insignificant relationship of the oxidases (PPO and POX) with PHOS and PMN at 4 weeks (Table 1.1D, Fig. 1.4, see supplementary Fig. 1.3 for 3D plots). At 4 weeks, each of the soils showed similar patterns where green waste amended soils had the highest hydrolase C activity, followed by compost or biochar and unamended soils. The pattern for oxidase C activity was the opposite for amended soils, where compost or biochar amended soils had higher oxidase C activity than green waste amended or unamended soils.

Based on NMDS, overall microbial activity in the green waste amended soils was least similar to unamended soils, whereas overall microbial activity in compost and biochar amended soils were more similar to unamended soils at 4 weeks (Fig. 1.3B). At 12 weeks, overall microbial activity became more distinct among the differently treated soils in each soil. At the same time, overall microbial activity in green waste amended soils became more like that of the unamended soils as compared to the activity at 4 weeks, while overall microbial activity in compost amended soils remained similar to that at 4 weeks (Fig 1.3B).

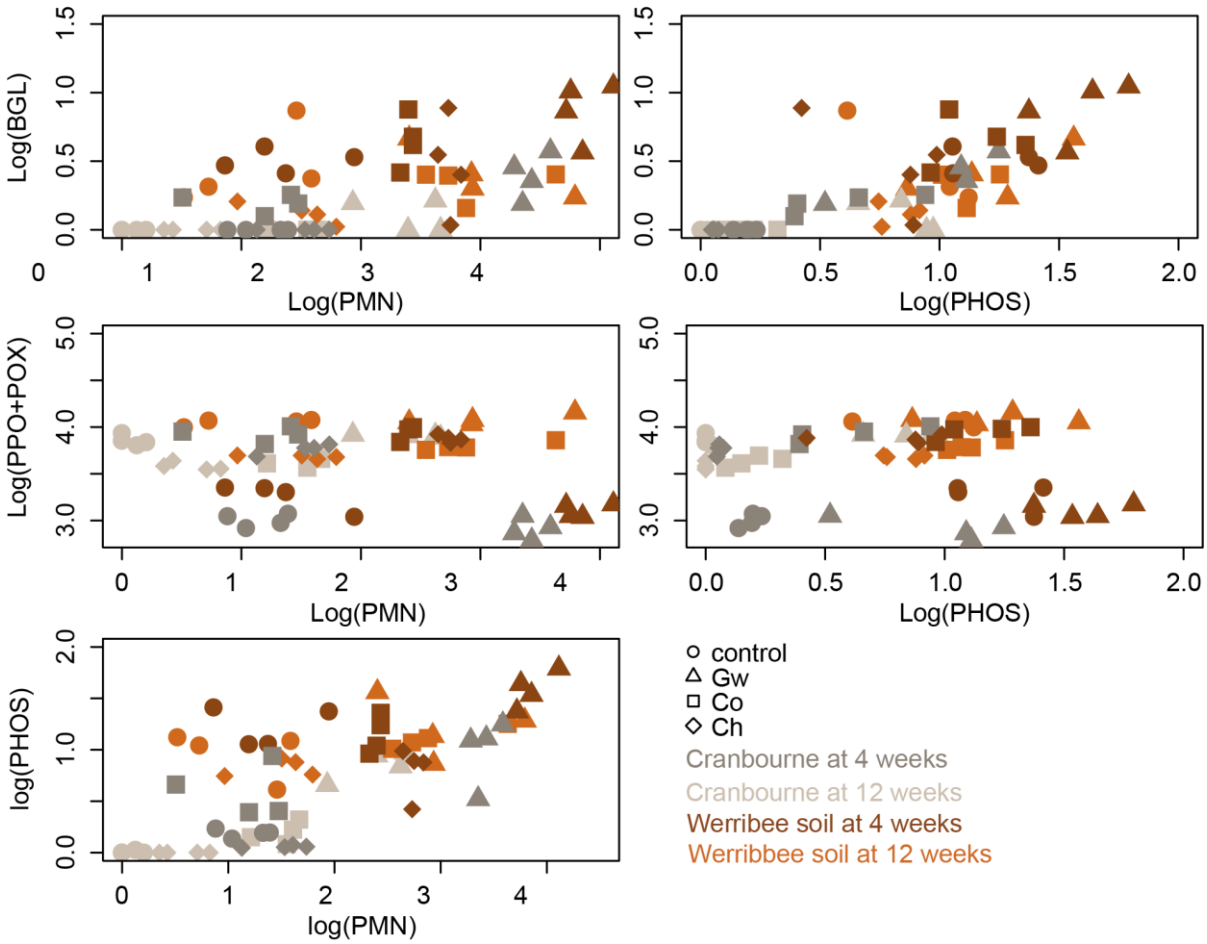

Fig. 1.4 Microbial C activity for hydrolase BGL or oxidase PPO+POX in relation with microbial N activity (PMN) and microbial P activity (PHOS) for 4 and 12 weeks. Correlation between microbial C,N and P activities analysed using Kendall τ (table 1.1D). See supplementary Fig. 1.3 for 3D plot equivalents.

We analysed the mean ratio of microbial C:nutrient activities of controls and treatments combined. The ratio of log(BGL):log(PMN) averaged 0.18 ± 0.03 (se); the corresponding log(BGL):log(PHOS) averaged 0.38 ± 0.07. The ratio of log(PPO+POX): log(PMN) averaged 2.09 ± 0.24; the corresponding log(PPO+POX):log(PHOS) averaged 13.4 ± 3.76. By 12 weeks, hydrolase C activity has declined in all amended soils, with the greatest decline found in green waste amended soils. At the same time, oxidase C activity has increased in green waste amended soils but oxidase C activity remained about the same in compost or biochar amended soils. Correspondingly, the ratio of log(BGL):log(PMN) averaged 0.11 ± 0.03(se); the log(BGL):log(PHOS) averaged 0.24 ± 0.06. The ratio of log(PPO+POX):log(PMN) averaged 4.47 ± 1.13; the corresponding log(PPO+POX):log(PHOS) averaged 12.8 ± 5.70.

Relationships between functional stoichiometry, resource stoichiometry and mineral nutrients

The ratios of log(BGL):log(PMN) at both sampling times were negatively correlated with soil C:N ratios and the ratios of log(BGL):log(PHOS) were negatively correlated with soil C:P ratios at both sampling times (Table 1.1B). There were also some weaker positive correlations between functional C:nutrient ratios to soil C:nutrient ratios when oxidase C activity (PPO+POX) was examined instead of hydrolase C activity (BGL). Functional C:nutrient ratios correlated only with input C:nutrient ratio for the ratios of log(PPO+POX):log(PMN) at 4 weeks.

Based on RDA, the variations in microbial activity among the treatments were largely due to differences in PMN and PPO activity at 4 and 12 weeks, in both soil types (Supplementary Fig. 1.1B). At both times, soils amended with green waste were generally characterised by higher PMN and lower PPO activity as compared to unamended soil or soil

amended with compost or biochar, which were generally characterised by higher levels of PPO and lower PMN (Supplementary Fig 1.1B). RDA identified soil NO_3^-, soil NH_4^+, initial C content, soil C:P ratio and soil type as important for explaining 46.9% of total variance in microbial activity among treatments (Supplementary Fig. 1.1B). Although the addition of all of the organic amendments to both soils generally resulted in increase in available nutrients, the increase due to compost was generally high and sustained over time compared to green waste and biochar in both the Werribee and Cranbourne soils (see supplementary table S1.1).

1.4. Discussion

In this study, we explored the utility of ecological and functional stoichiometry theories for understanding soil microbial transformation of organic amendments. We also examined key relationships between soil microbial community composition, biomass and activity with resource elemental composition and nutrient availability. We found that the addition of organic amendment lead to a distinct microbial composition with similar constant ratios of C:N:P activities. We observed generally consistent negative correlations between the functional ratio of log(BGL):log(nutrient) and soil C:nutrient, and inconsistent weak positive correlations between the functional ratio of log(PPO+POX):log(nutrient) and soil C:nutrient at both sampling times. The constancy of the functional stoichiometry indicates that microbial composition can be different but there is a limit to their response. We found that functional stoichiometry correlates well with soil stoichiometry and poorly with input stoichiometry. These findings were further supported with results of a redundancy analysis that identified variations in initial C content, mineral N, ratios of soil C:nutrient and site as drivers of variations in microbial community composition and activity under different organic amendments.

Relationship between microbial community composition, structure and resource stoichiometry

By analysing PLFA profiles of the treated soils, we found that microbial community composition and structure were distinct with different organic amendments and these variations in composition and structure with organic amendment persisted over time. The bacteria:fungal ratio was positively correlated to input C:nutrient ratio at 4 weeks and negatively correlated to soil C:N ratio at 12 weeks, suggesting a turnover in the microbial community composition as the resource base changed. For example, there were shifts in the bacteria:fungal ratio in the compost amended soils from 4 to 12 weeks but this shift in bacteria:fungal ratio occurred in opposite directions for Cranbourne and Werribee soils. It is possible that these shifts in relative abundances are associated with competitive interactions between the different microbial guilds present in each soil (Fontaine et al., 2003; Moorhead and Sinsabaugh, 2006). It is also important to consider the changes in microbial community composition due to inclusion of microbes originating from the organic amendment. Based on NMDS analysis, we found that the PLFA composition of the compost or raw green waste amended soils clusters with the PLFA composition of the original input, particularly at 4 weeks (data not shown). Additionally, the negative relationship between bacteria:fungal ratio and soil C:N ratio at 12 weeks generally agrees with previous findings that soils with higher C:N ratio tend to become increasingly dominated by fungi (Fierer et al., 2009; Waring et al., 2013). As bacteria generally require more N per unit biomass and have lower carbon use efficiency than fungi at higher resource C:nutrient stoichiometry, stoichiometric and physiological constraints may favour fungi over bacteria in soils with higher C:N (De Deyn et al., 2008; Keiblinger et al., 2010). Given that we have not measured microbial elemental C content, this remains to be confirmed.

Interestingly, we found that resource stoichiometry better accounted for variations in soil microbial community composition than soil microbial activities. Based on the RDA, ratios of soil C:N and soil C:P were important variables in explaining the variations in soil microbial community composition as shown by the length of the vector (see supplementary data). These ratios of soil C:nutrient act on different axes and are associated with the change in microbial composition from 4 to 12 weeks. Resource quality is thought to affect microbial element use efficiency (Manzoni et al., 2012; Mooshammer et al., 2014), and our results suggest the microbial community element use efficiency is flexible in response to resource dynamics due to shifts in microbial community composition. These results support Fanin and colleagues' (2013) hypothesis that the changes in resource ratio leads to changes in community composition rather than changes in stoichiometry of the same community. This view on the role of resource competition in the organisation of soil microbial community is reminiscent of Hutchinson's (1961) paradox of the plankton and Tilman's (1982) resource competition and resource-ratio theory, which describe how it is possible for many competing species to co-exist. On the other hand, the ratio of soil C:P or soil C:N explained less than one percent of the overall variations in soil microbial activities (data not shown). Additionally, its importance relative to the other explanatory variables (initial C content and mineral N) was lower as seen in the length of the vector (see supplementary data). It is likely that microbial activities are more dynamic and reflects the immediate conditions while microbial composition reflects longer term soil organic matter dynamics (Grandy et al., 2007).

Relating functional stoichiometry to resource stoichiometry

Our measures of microbial C, N and P activities showed similar relationships where the increase in one is generally correlated with the increase in the other. Such relationships

were strong between hydrolase BGL with N and P activities but inconsistent and weaker in the oxidases PPO and POX with N and P activities. The positive correlations observed between the functional ratios of log(PPO+POX):log(nutrient) and soil C:nutrient, and the negative correlations between functional ratio of log(BGL):log(nutrient) and soil C:nutrient indicate that microbes respond to differences in resource quality by shifting their activities accordingly. This coupling of microbial activities makes sense as enzyme production is energetically costly and mechanisms to synchronise optimal enzyme activity is crucial to minimise entropy. These results also indicate that there is great capacity to response to the different organic amendments in both soils, but this response is constrained by stoichiometry, as proposed by Sinsabaugh and colleagues (2009; Sinsabaugh et al., 2008). Based on these results and our findings in Ng et al. (2014; see Chapter 2), measures of microbial activity may serve to extend functional stoichiometry from nutrient availability to carbon composition as proposed by Sinsabaugh and Shah (2011). Previous studies have found oxidases PPO and POX to be more variable compared to hydrolase BGL (Sinsabaugh et al., 2008; Sinsabaugh and Shah, 2011). Although we found that PPO and POX activity inconsistently associate with that of other hydrolases, it is possible that the sequential breakdown of more recalcitrant C sources by oxidases followed by increased hydrolysis of more labile products leads to a temporal coupling of microbial oxidative and hydrolytic enzyme activities (Moorhead et al., 2013a; Šnajdr et al., 2011). This is a subject for further investigation.

We had expected functional stoichiometry to relate better to the input stoichiometry initially and, as the organic amendment decomposed, that functional stoichiometry would correlate better with soil stoichiometry, reflecting the change in resource for the soil microbial community. However, we found that the functional stoichiometry was more consistently correlated with the stoichiometry of the soil (i.e. soil C: nutrient ratios) than with the input stoichiometry, at both 4 weeks and 12 weeks after addition of the organic

amendment. As functional stoichiometry is a function of resource quality and the elemental requirements of the microbial biomass, these results suggest on one hand that the organic input was not necessarily the primary resource for the soil microbial community 4 weeks after input application. Microbial necromass has been found to be a substantial resource at any given time during litter decomposition (Kaiser et al., 2014). On the other hand, it could also indicate that the elemental requirements of the microbial biomass had changed, which is supported by the observed changes in the microbial community composition.

It has been proposed that that the C storage potential of a system depends on its ability to sequester more nutrients (Fontaine et al., 2004; Kirkby et al., 2011). This is supported by our observation that the amounts of C, N and P in the soils were highly correlated. As the stoichiometry indicates no saturation in the nutrient cycling in this experiment (i.e. there was no apparent levelling off in terms of functional or soil C: nutrient ratios), there is the possibility to further increase C, N and P cycling through increasing the quantity and quality of organic amendments added to the soil (see Ng et al. 2014; Chapter 2).

Soil microbial biomass dynamics

The addition of chemically different organic amendments resulted in clear differences to the growth dynamics (measured as total PLFA) of the soil microbial community. Microbial biomass rapidly increased in both soils receiving input of raw green waste, but the population was not sustained over the 12 week incubation period. In contrast, compost amended soil showed a lower, but sustained, increase in total microbial biomass over the 12 weeks, whilst biochar actually inhibited growth in the Werribee soil at 4 weeks. The fact that no correlation was observed between the input stoichiometry and the overall microbial biomass indicates that the size of the microbial biomass is not strongly dependent on the stoichiometry of the

input at 4 or 12 weeks after the incorporation of the organic inputs. It is proposed that microbial growth is limited by C:N ratio of the bioavailable dissolved organic matter, which is derived from a mixture of complex plant-derived and microbial-biomass derived pools (Kaiser et al., 2014). In this study, we found a good correlation between microbial biomass and NH_4^+ or NO_3^-, which indicated the influence of available nutrients from the overall input-soil mix on microbial biomass.

Study limitations and considerations for future work

Although this study demonstrates the potential for stoichiometry theories to contribute to understanding organic matter dynamics, limitations to both this study and the framework are apparent. First, redundancy analysis indicated that as much as 47% of microbial community composition and activity can be explained by variation in the initial C content, mineral N (NO_3^-, NH_4^+), site (or soil type) and ratios of soil C:nutrient. However, unmeasured properties of the organic amendment and/or the environment still account for another 50% of the variation. In Ng et al. (2014; see chapter 2), we also found that carbon composition of the organic amendment and subsequent changes in bulk soil carbon composition due to the organic input are also important drivers of microbial community composition and activity. As shown previously, management practices not related to organic matter stoichiometry also account for a large proportion of the variation in microbial composition (De Vries et al., 2012; Kallenbach and Grandy, 2011) and may be even more dominant in other soils not explored here. Additionally, the spatial scale and time scale of our study is relatively narrow. Future work needs to integrate these aspects to improve predictions and determine their relative importance.

Second, variations in technical and statistical methodologies between published studies may give rise to slightly different or contrasting results, hindering a consistent interpretation (see Chapter 5). For example, Moorhead et al. (2013b) found that the dynamics of soil mineral nutrients provided little explanation for the dynamics of their system's microbial biomass or enzyme activities, whereas our study suggested the opposite, particularly with regard to NH_4^+. We used PMN as a proxy measure of microbial N activity rather than the fluorescence-based β-N-acetyl-glucosaminidase assay used by other authors including Moorhead et al (2013b). PMN is an integral measure of organic-N-cleaving enzymes over the period of one week, rather than an activity "snapshot", and probably contributed to the greater association we observed between microbial activity and nutrients. Our use of a multivariate approach, compared to the individual correlations used by Moorhead and colleagues may have further reinforced these differences. Finally, microbial biomass C was measured by fumigation extraction in Moorhead et al. while we used total PLFA as a measure of microbial biomass. PLFA turnover rate is determined by environmental conditions and therefore interpretation of microbial abundance is complex (Frostegård et al., 2011; Zelles et al., 1992). There is need for research to identify the best approaches and standards developed for ease of cross-study comparisons.

1.5. Conclusions

In conclusion, we observed that organic amendments can alter the soil microbial community size, composition and structure. Microbial community PLFA composition was distinct for each soil and organic amendment. Microbial activity was also strongly influenced by organic amendment. Furthermore, functional ratios of C:nutrient were generally constant,

indicating that microbial composition can be different but that there are stoichiometric limitations to their responses.

The results presented here highlight the potential for organic amendments to play a major role in sustainable biologically regulated nutrient supply systems, and the potential to monitor these changes using a stoichiometric approach. By linking functional stoichiometry to resource stoichiometry and soil microbial composition, we have gained new insights into key relationships and constraints between regulators of the transformation of organic matter in the soil. Much of the information used to develop enzyme-based models to date was gleaned from multiple studies and result in uncertainty about key relationships (Moorhead et al., 2013b). This study provides a form of "ground truthing" to examine the key relationships between soil microbial community composition, biomass and activity with resource elemental composition and nutrient availability. The results from our study provide indications that some key features of the soil system are robust and can be captured by cross-system analyses. Given the time and spatial scale of our study, similar studies with longer time scale and across larger spatial scales will confirm if this pattern is effectively transferred across hierarchies.

1.6. Acknowledgement

This work was supported by funding from the Monash Sustainability Institute Australia, Brown Coal Innovation Australia and the Australian Research Council (ARC). Experimental set-up, monitoring and chemical analysis was funded by the Department of Environment and Primary Industries, Victoria. The green waste used in this study was kindly supplied by SITA Australia. TRC also thanks ARC for the award of a Future Fellowship (FT120100463).

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Supplementary table S1.1. Chemical properties (mean ± se) of OA and treatments at each sampling time. Samples refer to Gw = green waste, Co = compost, Ch = biochar, We = Werribee unamended soil, Cr = Cranbourne unamended soil, WeGw = Werribee soil + green waste, CrGw = Cranbourne soil + green waste, WeCo = Werribee soil + compost, CrCo = Cranbourne soil + compost, WeCh = Werribee soil + biochar, CrCh = Cranbourne soil + biochar.

	pH (H_2O)	Total N (g/ 100g)	Total C (g/ 100g)	Total P (mg/ kg)	NH_4^+ (mg/ kg)	NO_3^- (mg/ kg)	P-$CaCl_2$ (mg/ kg)
Gw	6.85 (0.07)	1.70 (0.03)	24.1 (0.3)	2726 (84)	75.5 (3.2)	0.1 (0.1)	67.23 (4.96)
Co	7.63 (0.03)	1.84 (0.02)	19.6 (0.2)	3660 (79)	2.5 (0.1)	331 (6)	74.53 (1.72)
Ch	10.50	1.16 (0.01)	24.4 (0.2)	3859 (64)	2.0 (0.7)	2.0 (0.4)	0.28 (0.02)
Week 4							
We	7.79 (0.01)	0.22 (0.00)	2.16 (0.02)	749 (9)	3.9 (0.2)	136 (1)	5.57 (0.04)
WeGw	7.88 (0.03)	0.26 (0.00)	2.69 (0.02)	860 (74)	5.1 (0.1)	95 (8)	6.10 (0.71)
WeCo	7.73 (0.00)	0.34 (0.01)	3.26 (0.02)	1010 (9)	4.9 (0.1)	180 (4)	8.04 (0.05)
WeCh	8.01 (0.01)	0.26 (0.00)	2.91 (0.04)	861 (15)	3.1 (0.1)	131 (2)	6.16 (0.33)
Cr	7.78 (0.01)	0.05 (0.00)	0.71 (0.01)	98 (3)	2.1 (0.1)	22 (1)	0.57 (0.05)
CrGw	7.89 (0.01)	0.10 (0.00)	1.41 (0.07)	164 (5)	3.0 (0.1)	10 (2)	2.55 (0.17)
CrCo	7.85 (0.03)	0.150 (0.01)	1.95 (0.07)	333 (20)	2.9 (0.1)	74 (4)	3.91 (0.19)
CrCh	8.38 (0.02)	0.09 (0.00)	1.65 (0.04)	247 (10)	0.6	24	2.04 (0.03)
Week 12							
We	7.77 (0.02)	0.23 (0.00)	2.11 (0.01)	756 (7)	2.9 (0.4)	184 (9)	5.42 (0.07)
WeGw	7.80 (0.04)	0.28 (0.00)	2.54 (0.02)	821 (8)	3.9 (0.2)	162 (18)	7.58 (0.37)
WeCo	7.42 (0.01)	0.34 (0.01)	3.21 (0.07)	967 (40)	3.0 (0.2)	240 (6)	9.19 (0.29)
WeCh	7.74 (0.01)	0.26 (0.00)	2.89 (0.04)	882 (13)	1.7 (0.1)	178 (19)	6.46 (0.12)
Cr	7.85 (0.00)	0.06 (0.00)	0.72 (0.04)	109 (5)	1.6 (0.2)	35 (1)	0.58 (0.02)
CrGw	7.84 (0.02)	0.12 (0.00)	1.23 (0.03)	186 (14)	2.8 (0.2)	67 (5)	3.19 (0.24)
CrCo	7.53 (0.01)	0.19 (0.02)	1.81 (0.07)	381 (33)	3.0 (0.2)	118 (2)	5.33 (0.24)
CrCh	8.19 (0.01)	0.10 (0.01)	1.58 (0.07)	250 (12)	0.7 (0.1)	33 (1)	2.33 (0.03)

Table S1.2. Biological properties (mean ± se) at 4 and 12 weeks. Units for PHOS, BGL, PPO, POX = μmols/ h/ g soil; PMN = μg NH_4^+/ g soil. Samples refer to We = Werribee unamended soil, Cr = Cranbourne unamended soil, WeGw = Werribee soil + green waste, CrGw = Cranbourne soil + green waste, WeCo = Werribee soil + compost, CrCo = Cranbourne soil + compost, WeCh = Werribee soil + biochar, CrCh = Cranbourne soil + biochar.

	PHOS	PMN	BGL	PPO	POX	MBN
Week 4						
We	2.45 (0.34)	3.15 (1.00)	0.66 (0.07)	23.0 (1.6)	2.30 (0.22)	40.1 (3.9)
WeGw	3.94 (0.43)	47.06 (4.54)	1.43 (0.25)	19.8 (0.7)	1.59 (0.14)	80.0 (14.0)
WeCo	2.20 (0.29)	10.02 (0.27)	0.94 (0.18)	47.5 (1.6)	3.49 (0.35)	159.9 (0.7)
WeCh	1.26 (0.25)	14.54 (0.61)	0.67 (0.29)	41.8 (0.9)	5.41 (0.61)	107.2 (5.5)
Cr	0.21 (0.02)	2.25 (0.38)	0.00 (0.00)	18.0 (0.8)	1.21 (0.11)	15.4 (2.7)
CrGw	1.79 (0.39)	29.55 (2.03)	0.49 (0.12)	16.1 (1.0)	1.34 (0.10)	57.6 (9.4)
CrCo	0.87 (0.25)	2.36 (0.61)	0.22 (0.04)	46.9 (1.9)	2.85 (0.07)	89.1 (13.7)
CrCh	0.06 (0.01)	3.59 (0.55)	0.00 (0.00)	37.5 (1.0)	4.60 (0.26)	31.1 (3.4)
Week 12						
We	1.68 (0.28)	2.23 (0.80)	0.62 (0.26)	45.9 (1.2)	10.52 (0.55)	64.1 (3.39)
WeGw	2.47 (0.50)	22.21 (7.27)	0.52 (0.15)	46.7 (1.2)	11.43 (0.56)	157.6 (5.43)
WeCo	2.05 (0.16)	19.91 (5.71)	0.41 (0.08)	40.0 (1.1)	3.56 (0.28)	50.3 (3.72)
WeCh	1.29 (0.10)	3.57 (0.72)	0.13 (0.04)	35.1 (0.7)	3.63 (0.36)	31.4 (2.82)
Cr	0.01 (0.01)	0.09	0.00 (0.00)	39.5 (1.5)	6.92 (0.48)	13.6 (3.40)
CrGw	1.37 (0.16)	10.51	0.11 (0.07)	39.5 (0.2)	9.15 (0.16)	135.6 (13.43)
CrCo	0.22 (0.06)	3.59	0.00 (0.00)	35.3 (0.7)	1.61 (0.40)	24.9 (4.39)
CrCh	0.00 (0.00)	0.82	0.00 (0.00)	33.2 (0.8)	1.77 (0.22)	10.6 (2.57)

Supplementary table S1.3. F and *p* values of ANOVA for soil microbial biomass, measured as total PLFA and bacterial: fungal ratio.

	OA $F_{3,24}$	Soil $F_{1,24}$	OA: Soil $F_{3,24}$
Total PLFA at 4 weeks	F = 270.7 *P* < 0.001	F = 118.1 *P*< 0.001	F = 5.1 *P* < 0.01
Total PLFA at 12 weeks	F = 51.8 *P* < 0.001	F = 84.0 *P* < 0.001	F = 4.1 *P* < 0.05
Bacteria: fungal ratio at 4 weeks	F = 144.3 *P* < 0.001	F = 121.7 *P* < 0.001	F = 47.6 *P* < 0.001
Bacteria: fungal ratio at 12 weeks	F = 50.7 *P* < 0.001	F = 46.0 *P* < 0.001	F = 14.4 *P* < 0.001

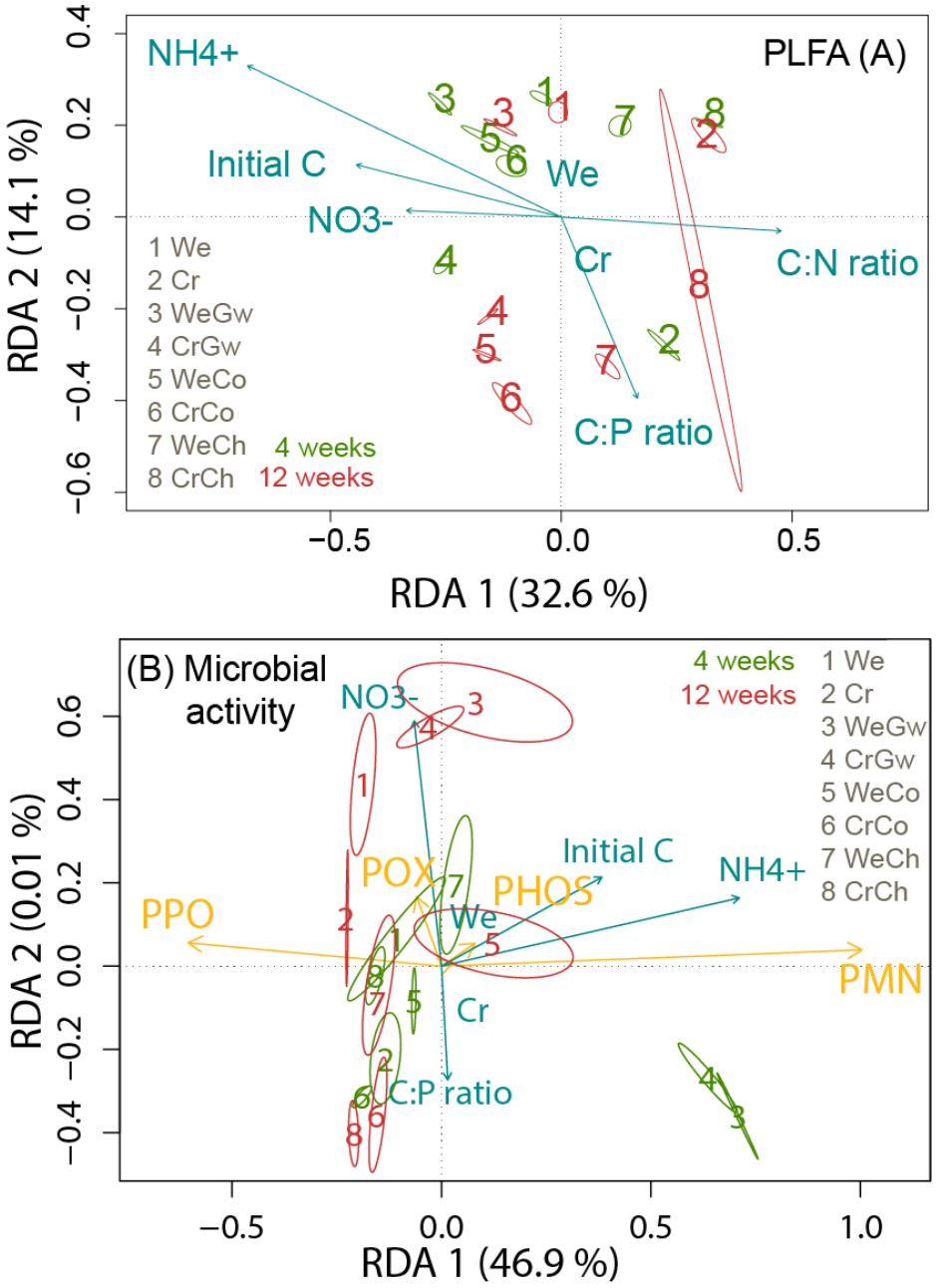
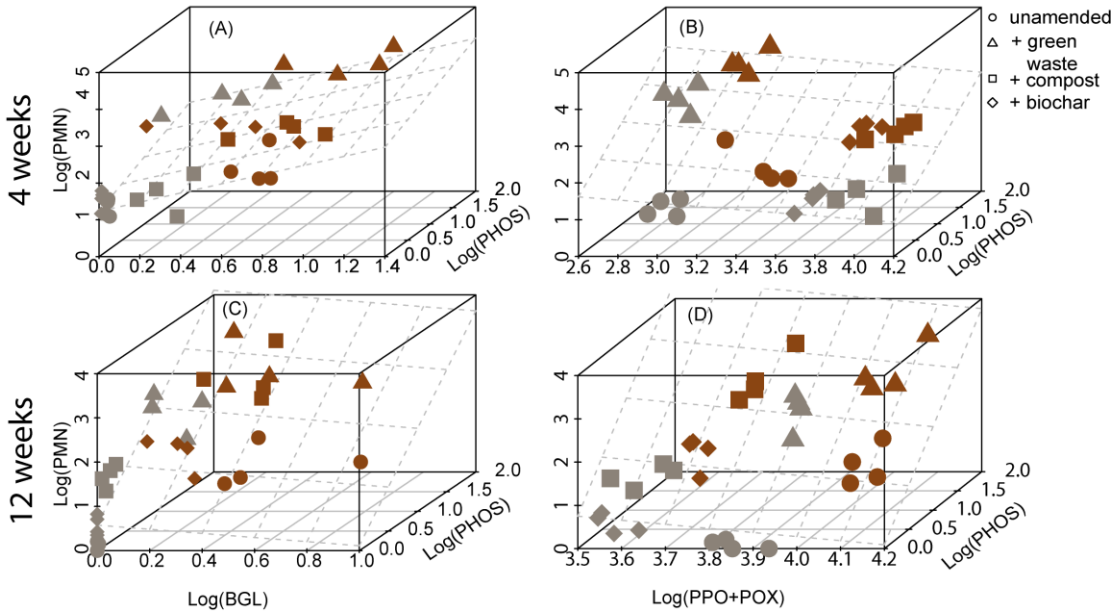


Fig. S1.1 RDA correlation plot showing variance explained by key edaphic properties. Microbial community based on (A) microbial PLFA composition and (B) microbial activity. BGL not shown on plots due to its low contribution to variations in microbial activity at 4 and 12 weeks. See fig. 1.2 for labels.



Supplementary Fig. S1.2 (A,C) Hydrolase BGL or (B,D) oxidase PPO+POX in relation with microbial N activity (PMN) and microbial P activity (PHOS) for 4 and 12 weeks. Brown = Werribee soil, grey = Cranbourne soil. Correlation between microbial C,N and P activities analysed using Kendall τ (table 1.1D).

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Setup study design, performed most of the lab work, analysed all the data and wrote the manuscript.	90

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Antonio Patti	Involved in study design, writing manuscript	2
Timothy Cavagnaro	Involved in study design, writing manuscript	2
Michael Rose	Microbial biomass analysis, writing manuscript	2
Kevin Wilkinson	Prepared organic amendments, fieldwork, experimental setup, writing manuscript	1
Cassandra Schefe	Involved in study design, writing manuscript	2
Ronald Smernik	^{13}C-NMR analysis and writing manuscript	1

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Soil Biology & Biochemistry 70 (2014) 54–61

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Does the chemical nature of soil carbon drive the structure and functioning of soil microbial communities?

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ARTICLE INFO

Article history:
Received 23 September 2013
Received in revised form
18 November 2013
Accepted 6 December 2013
Available online 18 December 2013

Keywords:
Soil enzymes
Carbon cycling
^{13}C NMR
PLFA
Microbial activity
Soil organic matter

ABSTRACT

The transformation of organic amendments (OA) in soil is in large part performed by soil microbial communities. These processes are strongly affected by the carbon composition of the OAs. We examined microbial community responses to three types of OA: green waste, composted green waste and pyrolysed green waste added to two contrasting agricultural soils. We investigated the relationship between the soil carbon composition (as determined by ^{13}C-solid state NMR), microbial community composition (as determined by phospholipid fatty acid analysis) and microbial activity (as determined by soil enzyme assays). We found that alkyl-C, O-aryl-C, aryl-C and carbonyl-C were able to explain most of the variations (≥50%) in soil microbial community composition and activity. Aryl-C content (reflecting relatively stable carbon forms) strongly influenced microbial composition, while carbonyl-C content (reflecting relatively labile carbon forms) strongly influenced the microbial activity. Our results confirm that there is a tight relationship between carbon composition and soil microbial community composition and function. Results are discussed in the context of examining the relationship between carbon forms, microbial community composition and activity following the addition of different OAs to the soil.

1. Introduction

There is more carbon in the soil than in all terrestrial plants and the atmosphere combined. Thus soils play a major role in regulating the global carbon cycle (Kleber, 2010; Lal, 2010). Carbon is present in the soil in many different forms and compounds that vary greatly in their chemical nature (Baldock et al., 2004). These differences in carbon composition play a major role in microbial-mediated soil carbon cycling. If we are to increase soil carbon stocks as a means of mitigating climate change, we must not only consider the amount of carbon present in the soil, but also the forms in which it is present, and the impact this has upon soil microbes involved in soil carbon cycling. With the great diversity of microbes and complexity of carbon forms present in soil, this is a challenging issue that is yet to be fully resolved.

The conversion of natural lands to agricultural production has led to a significant reduction in soil carbon levels (Lal, 2011). With 38% of the earth's terrestrial surface being used for agricultural production (FAOSTAT, 2010), the potential to mitigate rising atmospheric CO_2 levels through soil carbon sequestration in agroecosystems is large; the global soil carbon sequestration potential of agricultural lands has been estimated at 2.1 billion tons C/yr (Lal, 2010). The addition of organic amendments (OA) to soil has been suggested as an option for supplying nutrients to support agricultural production, while increasing soil carbon levels (Quilty and Cattle, 2011). Furthermore, where OA are derived from waste streams, such as municipal green waste or animal manure, there is the added benefit of recycling of nutrients and carbon that would otherwise be 'lost'. Despite being rich in carbon and nutrients, where the addition of OA to the soil yields an increase in soil carbon, usually only a small percentage (<20%) of this carbon is incorporated into the stable soil organic matter (SOM) pool (Sanderman et al., 2010). In some cases, the addition of OA to the

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http://dx.doi.org/10.1016/j.soilbio.2013.12.004

soil has actually resulted in a reduction in soil carbon levels due to priming effects (Fontaine et al., 2004). If we are to realize the full potential of OA to increase soil carbon levels while providing nutrients to plants, we must understand the processes that drive OA transformations in soil.

Soil organic matter transformation is primarily carried out by soil microbes, via processes including decomposition, polymerisation, protection and immobilisation (Jastrow et al., 2007; King, 2011; Sinsabaugh, 2010). These processes are mediated via the production of enzymes by soil microbes. Consequently, soil microbes are considered proximate controllers of soil organic matter (SOM) transformations. Given the chemical complexity of SOM, a wide range of soil enzymes are involved in organic matter transformation. For example, hydrolytic enzymes, such as β-glucosidase and phosphatases, catalyse specific steps in mineralisation of carbon and phosphorus (Shi, 2010), whereas oxidative enzymes, such as phenol oxidases, act more generally on phenolic-containing compounds including lignin, humus and polyphenols (Sinsabaugh, 2010). Thus, quantification of soil enzyme activities provides important mechanistic and functional knowledge of SOM transformation processes.

Due to the heterogeneity in the sources of organic inputs from one ecosystem to another, SOM composition can be highly variable. The composition of SOM affects its residence time as it influences biological stabilisation, physical protection and the energy required for SOM breakdown (Ekschmitt et al., 2005; King, 2011; Kögel-Knabner et al., 2008; Schmidt et al., 2011). For example, as the decomposition of SOM progresses, the relative proportion of aromatic structures in SOM has been reported to increase (Gressel et al., 1996; Pedersen et al., 2011), as less stable structures are degraded. Phenolic compounds have been correlated with the antioxidant capacity of soils that neutralises free radicals, and therefore, protects organic matter from oxidation (Rimmer and Abbott, 2011). Aromatic compounds have also been implicated in hydrophobic protection of SOM (Spaccini and Picollo, 2012). The complexity of SOM composition is especially relevant in the context of OA transformations given the tremendous chemical heterogeneity and complexity of OA. This in turn can also strongly affect the fate of the OA in the soil.

Many OA undergo some form of 'pre-processing' prior to application to the soil; for example, organic matter is often composted to eliminate viable propagules of pests and pathogens, and further stabilise the organic matter it contains. Similarly, pyrolysis, that is the elevated thermal decomposition of organic matter in the absence of oxygen, is also increasingly being used as a means of stabilising OA prior to their addition to the soil. Given the differences in the nature of these different processes, they are expected to affect the chemical nature of the resulting OA. For example, we would expect that OA derived from pyrolysis processes to have a greater proportion of aromatics than their parent material (Lehmann et al., 2011). In turn, such changes in the properties of the OA are likely to have direct effects on the soil microbial community composition and activity, and indirect effects through changes in the interaction of the OA with the abiotic environment (Bastida et al., 2008; Cross and Sohi, 2011; Pérez-Piqueres et al., 2006; Singh et al., 2012). Therefore, we contend that if we are to understand the fate of OA in the soil, we need to take into consideration OA, how it changes SOM chemistry and how this affects microbial metabolism (Cebrian, 1999; Manzoni et al., 2008; Moorhead and Sinsabaugh, 2006; Schimel and Weintraub, 2003).

One approach to studying the relationship between organic matter chemistry and microbial metabolism is to amend soil with known or labelled substrates, alone or in combination (e.g. Orwin et al., 2006). While this approach can yield detailed information about the turnover of specific compounds or groups of compounds, it can be difficult to use such results to make inferences about the behaviour of complex SOM pools. An alternative approach is to describe the chemical nature of soil carbon pools and OA inputs in the soil environment, e.g. by using solid-state ^{13}C nuclear magnetic resonance spectroscopy (^{13}C NMR). As ^{13}C NMR provides overall carbon characterisation of the SOM, it is a powerful tool to link changes in the nature of SOM to soil microbial community composition and/or functions. A number of studies have linked carbon forms to microbial community composition (e.g. Pascault et al., 2010), or carbon forms and microbial activity (e.g. Alarcón-Gutiérrez et al., 2008; Flavel and Murphy, 2006; Pane et al., 2013). However, to our knowledge, there have been few studies of OA transformation where changes in the chemical nature of carbon containing compounds in the soil, brought about by adding organic amendment and soil microbial community composition and activity, were simultaneously quantified (e.g. Moorhead and Sinsabaugh, 2006; Šnajdr et al., 2011; Wickings et al., 2012). If OA are to become a reliable soil carbon input, such knowledge will be essential.

Here, we report findings of an experiment in which we added three OA sourced from the same municipal green waste but treated to produce three materials of different chemical stability – raw green waste, composted green waste and pyrolysed green waste (biochar) – to two contrasting soils. We then measured differences in the soil carbon composition, the soil microbial community composition and activity after 12 weeks of incubation. Specifically, we hypothesise that the changes in chemical nature of soil carbon as a result of the addition of OA would be reflected in the soil microbial structure and activity.

2. Materials and methods

2.1. Site, experimental design and sampling

We set up a microcosm-based incubation experiment using two soils. The Cranbourne soil (Cr) was collected from a horticultural farm in Cranbourne, Australia (38°11′ S 149°19′ E). It is a semiaquic Podosol of loamy sand texture with a pH of 7.79 (H_2O), a C:N ratio of 13 and contains 1.3% organic matter. The Werribee soil (We) was collected from a horticultural farm at Werribee, Australia (37°53′ S, 144°40′ E). It is strongly a dispersive (basaltic) red Sodosol of a slightly sodic light clay texture with a pH 7.79 (H_2O), a C/N ratio of 9.1 and contains 3.9% organic matter. Both soils were collected from 0 to 10 cm depths, air dried and sieved to 2 mm. To 300 g of each soil, OA were added separately as raw green waste (Gw), composted green waste (Co) and green waste biochar (Ch) and thoroughly mixed in at rates that aimed to increase total soil C by 1%. This series of OA were selected as they were expected to have increasingly more stabilised carbon and nutrient pools. For details on the composting and pyrolysis, see Supplementary information. Soils without OA were used as controls in the experiment. The soil microcosms were maintained at moisture between −30 and −40 kPa, and incubated at 25 °C in the dark. Each treatment was replicated four times, and sampled at 12 weeks. The soil from each replicate was then divided into three sub-samples to be stored at 4 °C for microbial activity assays, at −20 °C for phospholipid fatty acid (PLFA) analysis, and air-dried for chemical analysis.

2.2. Carbon characterisation by ^{13}C NMR

Sub-samples from the four replicates of each treatment were combined for ^{13}C NMR analysis. Solid-state ^{13}C cross polarization (CP) NMR spectra were acquired with magic angle spinning (MAS) at a ^{13}C frequency of 50.33 MHz on a Bruker 200 Avance spectrometer. Samples were packed in a 7 mm diameter cylindrical zirconia rotor with Kel-F end-caps, and spun at 5 kHz. Spectra were

acquired using a ramped-amplitude cross polarization (CP-ramp) pulse sequence, in which the ^{1}H spin lock power was varied linearly during the contact time. A 1-ms contact time and a 1-s recycle delay were used and 30,000 transients were collected for each spectrum. All spectra were processed with a 50 Hz Lorentzian line broadening. Chemical shifts were externally referenced to the methyl resonance of hexamethylbenzene at 17.36 ppm.

All spectral processing was completed using Bruker TopSpin 3 software. Empty rotor background signals were subtracted and the resultant spectra were integrated across the following chemical shift limits to provide estimates of broad carbon types: 0–45 ppm (alkyl C), 45–60 ppm (N-alkyl C), 60–110 ppm (O-alkyl C), 110–145 ppm (Aryl C), 145–165 ppm (O-aryl-C), and 165–215 ppm (Carbonyl C). Signal intensity found in spinning side bands was allocated back to their parent resonances according to the calculations presented by Baldock and Smernik (2002).

2.3. Microbial activity

We assayed the potential activity of four enzymes in the soil samples, here-on referred to as microbial activity. β-glucosidase (BGL), phosphatase (PHOS) and polyphenol oxidase (PPO) activity were determined according to methods modified from Allison and Jastrow (2006). Peroxidase (POX) activity was assayed using a method modified from Frey et al. (2000) and Johnsen and Jacobsen (2008). We incubated 0.5 ml of homogenised soil slurry (1 g soil in 50 ml sterile H_2O) with 0.5 ml of substrate solution (see Table 1 for substrates and incubation times). 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (100 mM, pH 7) was used to make up substrate solutions for all assays except for the POX assay, for which acetate buffer was used (100 mM, pH 5). We used buffer of pH 7 where possible to match our soil pH. All assays included a background soil control and a substrate control. A background soil control contained soil slurry and buffer. A substrate blank contained substrate solution and sterile H_2O. Absorbance was measured at 405 nm except for the POX assay, which was measured at 450 nm. Microbial N activity was measured using potentially mineralisable nitrogen (PMN) as a proxy measure in 5 g aliquots of soil using anaerobic incubation (Waring and Bremner, 1964). The ammonium (NH_4^+) was extracted with 2 M KCl and measured colorimetrically following Forster (1995).

2.4. PLFA analysis

We extracted PLFA following the procedures of Bossio and Scow (1998) with slight modification. Lipids were extracted from 4 g of lyophilised soil using 15.6 ml citrate buffer (0.15 M, pH 4.0): $CHCl_3$: methanol (0.8:1:2 v/v/v) mixture. Samples were shaken for 1 h at room temperature then centrifuged for 10 min at 1900 × *g*. The supernatant was transferred into a clean glass tube. A further 11.7 ml citrate:$CHCl_3$:methanol (0.9:1:2 v/v/v) mixture was added to the soil pellet. The samples were shaken and centrifuged, and the supernatant was isolated and combined with the first supernatant. A further 13.3 ml of citrate: $CHCl_3$ (0.9:1 v/v) mixture was added to the pooled supernatant. Samples were left overnight for phase separation, after which the aqueous layer was removed and the $CHCl_3$ layer evaporated under a stream of N_2. Each sample was redissolved in 2 ml of $CHCl_3$ and transferred to solid phase extraction cartridges for separation of lipid classes. An aliquot of 3 ml of $CHCl_3$ was added followed by 2 aliquots of 5 ml of acetone. These extracts were discarded. The phospholipid fraction was collected by extracting the cartridges with 5 ml of methanol; the methanol was then evaporated under N_2. For methanolysis, the phospholipid fraction was incubated at 37 °C for 20 min with 1 ml of a 1:1 mixture of methanol and toluene and 1 ml of methanolic KOH (0.2 M). The samples were neutralised with 0.3 ml acetic acid (1 M) and 2 ml of ultrapure H_2O. Two extractions were carried out with a mixture of 2 ml hexane: $CHCl_3$ (4:1 v/v) and the organic phases combined. The organic layer was collected and evaporated again under a stream of N_2. Each sample was resuspended in 200 μl of hexane containing methyl decanoate (0.005 mg/ml) and analysed using gas chromatography. The chromatography was conducted with a 30 m (5%-phenyl)-methylpolysiloxane column (Varian CP 3800), using He as a carrier gas, an FID detector, and a temperature program of 120 °C initial temperature, ramped to 220 °C at 4 °C/min, ramped to 325 °C at 20 °C/min, and held 325 °C for 8 min. Bacterial phospholipid markers of interest were i15:0, a15:0, 15:0, i16:0, 16:1ω7, i17:0, a17:0, 17:0cy, 17:0, and 19:0cy (cf with Frostegård and Bååth 1996, and references therein). Linoleic acid (18:2ω6,9) was used as an indicator of fungal biomass (Frostegård and Bååth 1996). Concentrations of fatty acid less than 0.1 ppm were treated as 0 and only fatty acids detected in >4% of treatment were included in the analysis; as a result a total of 21 PLFAs were included.

Table 1
Enzyme substrates and assay time.

Enzyme	Substrate	Assay time (min)
β-glucosidase (BGL)	5 mM *p*NP-β-D-glucopyranoside (Sigma N7006)	120
Acid phosphatase (PHOS)	5 mM *p*NP – phosphate (Sigma P4744)	120
Peroxidase (POX)	1 mM 3,3′,5,5′-tetramethylbenzidine or TMB (Sigma T2885)	10
Polyphenol oxidase (PPO)	50 mM pyrogallol (Sigma 254002)/50 mM EDTA	60

2.5. Calculations and statistical analysis

Standardised data were used for multivariate regression tree (MRT), non-metric multidimensional scaling (NMDS), redundancy analysis (RDA) and cluster analysis. The PLFA was standardised by dividing values of each individual fatty acid by the total PLFA. Microbial activities were standardised by chi-square transformation using decostand( ) function in *vegan* package. The MRT was carried out to examine the relationship between C forms and soil microbial community composition or microbial activities. This analysis involves a response (i.e. the composition or activity data set) and an explanatory data set as detailed in Legendre and Legendre (2012). It forms clusters of sites by repeated splitting of the response data set based on minimising dissimilarity of sites within each cluster. Selection of the best tree was based on cross validation and selecting the smallest tree within one standard error of the best following the method described by De'ath (2002). Unconstrained cluster analysis was comparable to MRT analysis and indicated that the explanatory variables accounted for most of the observed variations. NMDS analysis is an ordination method that plots dissimilar objects far apart in ordination space and similar objects close to one another. The NMDS was carried out to examine unconstrained patterns in microbial activity. For microbial community PLFA composition, cluster analysis was performed. A Bray–Curtis dissimilarity index was calculated using transformed PLFA data followed by Ward's minimum variance clustering. The PLFA clustering was then overlaid onto the ordination plot of the microbial activity as a minimum spanning tree. RDA was then carried out by constraining the microbial community composition or activity to ^{13}C NMR data as explanatory variables. Where the MRT and RDA analyses disagreed (in terms of variations explained), this informed us of (i) the

presence of interactions between the C forms which were not captured in RDA analysis since interactions were not included in the RDA analysis, and/or (ii) that the relationship between the C forms and microbial activity or composition was not linear as the RDA solution lies in linear space. All analyses were carried out in R 2.15.2 (R Core Team, 2012) using vegan (Oksanen et al., 2012) for RDA and NMDS, mvpart (Therneau et al., 2012) and MVPARTwrap (Ouellette and Legendre, 2013) for MRT.

3. Results

3.1. Linking microbial activity to microbial community composition

Patterns in soil microbial community composition and microbial activity could be explained by the type of OA added to the soil (Fig. 1). Specifically, the community composition was more similar between the two soils amended with the same OA than to their respective unamended soils; this is illustrated in the plot where a line connects the treatments based on the similarity in microbial community composition. For example, microbial composition in CrGw and WeGw were very similar, and microbial composition in Co or Ch amended soils were similarly strongly influenced by the OA. As for microbial activity, the type of amendment also separates the amended from the unamended soils; this is reflected in the relative location of the points in the ordination space, with those treatments closer to one another on the plot being more similar than to those further away in the plot. For example, microbial activity in CrGw and WeGw were more similar to each other than to their respective unamended soils. On the other hand, microbial activity in CrCo was more similar to that of CrCh, while microbial activity in WeCo was more similar to that of WeCh.

3.2. Linking microbial activity and microbial community composition to soil carbon forms

To further explore the relationship of carbon composition with soil microbial community composition and activity, we analysed our data using multivariate regression trees (MRT) and redundancy analysis (RDA). These analyses indicated that the relative content of alkyl-C, O-aryl-C, aryl-C and carbonyl-C explained most of the variation in soil microbial community composition and activity (Fig. 2). Furthermore, the differences in soil microbial community composition and activity due to the application of OA are associated with the relative importance of different C forms and the interaction and/or association among the C forms (Fig. 2A, C, see Supplementary Table S1 for C composition).

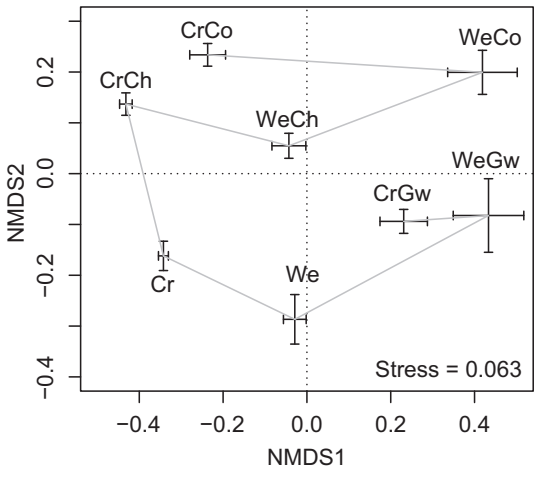

Fig. 1. NMDS plot (showing mean ± se) of microbial activity overlaid with PLFA composition, whereby the minimum spanning tree (line) shows the actual connection between the microbial community composition in one treatment to that in another treatment. We = Werribee soil, Cr = Cranbourne soil, Gw = green waste, Co = composted green waste, Ch = charred green waste (e.g. CrGw refers to Cranbourne soil amended with green waste). See Supplementary Table S2 for mean ± standard error values of microbial activity.

MRT analysis revealed that microbial activity could be separated into two main groups based on the relative abundance of carbonyl-C in the soil (46% of variation in microbial activities was explained by this split, Fig. 2A). Specifically, the microbial activities in WeCo and WeGw were associated with higher relative carbonyl-C content (>15.14%), compared to all other treatments. Further splitting of the remaining groups with lower relative carbonyl content (<15.14%) was delineated by aryl-C and alkyl-C. The MRT explained 79% of the total variation in microbial activity, of which 61% was explained by the first two splits in the tree. Thus, relative carbonyl-C content was the best predictor of soil microbial activity. Analysis of the data by MRT also allowed us to determine what and how much difference in the microbial activity was explained by the C forms. For example, PMN accounted for 58% of the variance in microbial activities, and 46% of this variance was explained by the tree, and 36% of this variance was explained by the first split (Table 2). POX and PPO largely determine the second split (aryl >/< 16.65), which explained 15% of the variance in microbial activity. Interestingly, total variance in BGL (4%) and PHOS (7%) was low and the tree managed to explain most of it (2% and 6%, respectively). Additionally, the MRT identifies potential interactions and/or associations among the C forms that result in the patterns observed for microbial activity. This is because each split of the MRT partitions data into independent subsets that are then further analysed independently (De'ath, 2002). For example, the interaction between carbonyl-C with aryl-C and alkyl-C produces differences in microbial activities.

RDA analysis of C forms and microbial activity largely showed similar patterns to those found in MRT analysis. For example, as in the MRT analysis, the microbial activity in the WeGw and WeCo treatments were closely, and positively associated with relative carbonyl content. We found that the relative content of carbonyl-C, aryl-C, O-aryl-C and alkyl-C explained 57% of total variation in microbial activity, of which 55.6% was explained by axis 1 and 2 (Fig. 2B). PPO activity was closely associated with O-aryl-C and aryl-C while BGL, PHOS, POX and PMN were more closely associated with carbonyl-C. Additionally, POX activity was also closely associated with alkyl-C.

The soil microbial composition was separated into two main groups by MRT analysis according to the aryl-C content, with higher relative content of aryl-C associated with the Ch and Co amended samples (Fig. 2C). Further splitting of the Ch and Co amended soil was determined by the relative contents of carbonyl-C, whereas unamended and Gw amended soils were further separated by O-aryl content. MRT explained 86% of the total variation in soil microbial community composition, of which 76% was explained by the first four splits. The variances of bacterial cy19:0 and fungi 18:2ω6 together comprise 60% of the total variance in microbial community composition; 50% of this variance was explained by the tree (Table 3). Bacterial cy19:0 dominated the first split (aryl >/< 17.0) while fungi 18:2ω6 dominated the fourth split (aryl >/< 16.6). The MRT also identifies potential interactions between the C forms that result in the patterns observed for microbial composition. For example, the interaction between aryl-C with carbonyl-C were most important for distinguishing microbial composition in Co and Ch amended soils while the interaction between aryl and O-aryl were more important for distinguishing microbial composition in Gw amended soils from unamended soils.

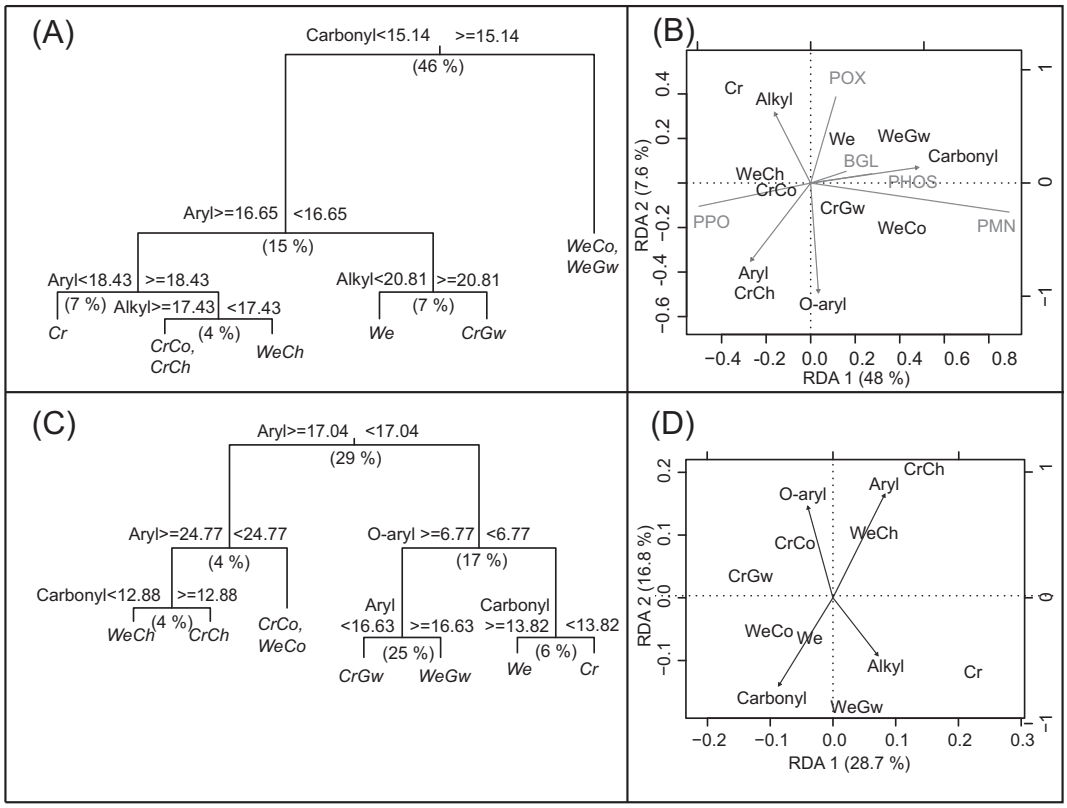

Fig. 2. Multivariate regression tree and redundancy analysis for the microbial community based on (A, B) microbial activities and (C, D) PLFA at 12 weeks. (A) The variation explained at each split of the MRT is shown as a percentage in parentheses in the body of each branch. Cross-validation error = 0.407, standard error of the tree = 0.098. (B) RDA correlation plot showing variance in microbial activities explained by C forms. (C) The variation explained at each split of the MRT is shown as a percentage in parentheses in the body of each branch. Cross-validation error = 0.237, standard error of the tree = 0.069. (D) RDA correlation plot showing variance in PLFA composition explained by C forms. We = Werribee soil, Cr = Cranbourne soil, Gw = green waste, Co = composted green waste, Ch = charred greenwaste (e.g. CrGw refers to Cranbourne soil amended with green waste). BGL = β − glucosidase, PHOS = phosphatase, PMN = potentially mineralisable nitrogen, PPO = polyphenol oxidase, POX = peroxidase.

RDA indicated that similar to microbial activity, carbonyl-C, aryl-C, O-aryl-C and alkyl-C explained a large portion (46%) of the total variation in the soil microbial composition (Fig. 2D). The microbial composition of Ch amended soils was associated with higher O-aryl-C and aryl-C compared to other treatments. While the soil microbial community composition of CrCo was similar to Ch amended soils in its association with higher O-aryl-C and aryl-C compared to other treatments, the microbial composition of WeCo was more similar to its unamended soil and both were associated with higher carbonyl-C and alkyl-C compositions.

4. Discussion

The patterns in soil microbial community composition and microbial activity observed here could be explained by the type of amendment added to the soil. Both RDA and MRT analysis of the data agree that soil carbon forms explain a large amount of the variation in the soil microbial activity and composition (46–86%). Based on the MRT, soil carbon forms had a larger influence on the microbial composition than the activity, as observed in the differences in variations explained. These results reinforce the important link between the nature of carbon in the soil, and the structure and function of soil microbial communities, in support of our hypothesis. Importantly, the patterns of the soil microbial community composition and activity responded differently to the soil carbon composition, highlighting the importance of the need to consider microbial community structure and function simultaneously rather than in isolation. Taken together, these results highlight the complexity of the relationship between SOM composition, soil microbial community composition, and soil functions, while providing new insights into the factors governing the fate of OA in agricultural soils.

Analysis of the data using MRT allowed us to use data on soil carbon composition to split the complex microbial compositional data in a manner that explained the majority of variation. For example, the first split in the soil microbial composition was driven by aryl-C and was largely due to the bacterial cy19:0 PLFA marker. Bacterial cy19:0 is associated mainly with Gram-negative bacteria (Zelles, 1997). These bacteria are generally known to utilise mainly simple C forms (Holding, 1960) yet they were found in this study to be more abundant in soils with higher relative aryl-C content. Aryl-C and O-aryl-C are aromatic compounds that are found in lignin, humic substances and biochars. They are associated with stabilised OM (Lehmann et al., 2011). But if we consider these results in the context of the observed microbial activities, then it appeared that these bacteria are present under conditions of higher PPO activity. Either these bacteria produce PPO to access the required carbon and nutrients, or they are riding very successfully on other microbes that do produce PPO.

In contrast, the fourth split in the tree for microbial composition was driven by aryl-C and was dominated by the fungal PLFA marker 18:2ω6,9. At this split, CrGw was distinguished from WeGw. The CrGw sample, which contained only marginally more aryl-C than WeGw, also had a greater proportion of fungal PLFA marker 18:2ω6,9. Given that ^{13}C NMR typically underestimates aryl-C and O-aryl-C (Smernik and Oades, 2000a, b), the differences in aryl-C

Table 2
Variance of soil microbial activity explained by the tree analysis.

Activity	Variance (%) of microbial activity explained by tree splits and whole tree						
	Carbonyl <15.14	Aryl <16.65	Aryl <18.43	Alkyl <20.81	Alkyl <17.43	Tree total	Activity total
POX	0.1	6.7	4.8	0.8	0.2	12.6	16.6
PPO	7.9	3.9	0.0	0.7	0.6	13.1	15.1
BGL	0.5	0.7	0.0	0.1	0.7	2.1	3.7
PHOS	1.7	1.4	0.4	1.9	0.1	5.5	6.8
PMN	35.6	2.4	1.7	0.3	5.4	45.3	57.8
Total activity variance	45.8	15.1	6.9	3.8	7.0	78.6	100.0

POX = peroxidase, PPO = polyphenol oxidase, BGL = β-glucosidase, PHOS = acid phosphatase, PMN = potentially mineralisable nitrogen.

content of the two may be greater than observed and this may explain why such marginal differences in aryl-C content leads to very different fungal PLFA marker 18:2ω6,9 composition. This is still surprising given the previously established relationship between fungi and recalcitrant SOM (De Boer et al., 2005; Garrett, 1951). Fungi are important producers of PPO and POX enzymes which degrade more recalcitrant aromatic compounds (Kirk and Farrell, 1987; Sinsabaugh, 2010). We had expected Co and Ch amended soils to have greater amounts of the fungal PLFA marker 18:2ω6,9; however, this was not the case, and is worthy of further investigation. Additionally, both Gw amended soils have relatively similar microbial activities. They are positively correlated with hydrolytic enzyme activities (BGL, PHOS) and carbonyl-C contents. Carbonyl-C is found in proteins and organic acids and they are important labile carbon forms. By combining these observations, we hypothesise that (i) bacteria may be playing a more important role in the degradation of recalcitrant C forms in these soils, and (ii) the dominant fungi composition in these Gw amended soils are copiotrophs.

Our results also indicated that presence of aryl-C favours the production of PPO (i.e. positive correlation), resulting in production of O-aryl-C. Such a combination of increasing PPO with aryl-C or O-aryl-C is expected of Co and Ch amended soils, and indeed our observations agreed with it. This is corroborated by our observations that soils amended with Co and Ch had higher metabolic quotient and therefore greater respiration per unit biomass compared to soils amended with Gw (Supplementary Fig. S2). In contrast, soil amended with Gw had high initial respiration rates and low metabolic quotient (Supplementary Fig. S2). Raw green waste, consisting mainly of O-alkyl-C, N-alkyl-C and alkyl-C contains a large pool of labile C. O-alkyl-C is found mainly in carbohydrates and lignin with smaller quantities in proteins and lipids (Baldock et al., 2004; Baumann et al., 2009). N-alkyl-C is found in proteins and lignin (Baldock et al., 2004). Alkyl-C is mainly found in lipids, waxes, cutins, suberins and lignin but is also found in proteins (Baldock et al., 2004; Baumann et al., 2009). The initial high respiration rates and low metabolic quotient provide support for the rapid consumption of labile C forms in the Gw amended soils by copiotrophs, which may explain the similarity of the microbial community composition of the Gw amended soils to the unamended soils 12 weeks after the addition of the OA. The subsequent succession of microbial community upon exhaustion of the easily assimilable C reflects the biotic legacy of the unamended soils.

As discussed above, the patterns of soil microbial community composition and activity clearly responded differently to the soil carbon forms. There are several possible explanations for these observed differences in the relationship between soil microbial composition and function to soil C forms. First, it may indicate that similar communities may not necessarily have similar functions. Strickland et al. (2009) found that harsher environment filters generate communities with more similar composition but dissimilar functional capabilities. Second, the weak relationship between composition and function may well reflect the temporal differences

Table 3
Variance of soil microbial PLFA composition explained by the tree analysis.

PLFA	PLFA variance (%) explained by tree splits and whole tree							
	Aryl <17.04	O-aryl <6.77	Aryl <24.77	Aryl <16.63	Carbonyl <13.82	Carbonyl <12.88	Tree total	PLFA total
Bacterial 14:0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1
Bacterial i15:0	0.6	1.3	0.2	0.0	0.1	0.7	2.8	3.3
Bacterial a15:0	0.1	0.6	0.1	0.0	0.0	0.2	1.0	1.2
Bacterial 15:0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1
2-OH 14:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3-OH 14:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Bacterial i16:0	0.2	1.7	0.0	0.0	0.0	0.1	2.1	2.3
Bacterial 16:1ω7c	0.6	0.5	0.0	0.2	0.0	0.0	1.4	1.6
Bacterial 16:1ω7t	0.6	0.1	0.0	0.2	0.0	0.0	0.9	1.0
Bacterial 16:0	2.4	1.0	0.7	2.3	0.2	0.5	7.0	7.5
Bacterial i17:0	1.0	0.8	0.1	0.1	0.0	0.0	2.0	2.2
Bacterial a17:0	0.4	0.4	0.0	0.0	0.0	0.0	0.8	1.0
Bacterial 17:0cy	0.0	0.1	0.0	0.2	0.0	0.1	0.4	0.6
Bacterial 17:0	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.2
2-OH 16:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fungi 18:2ω6,9	4.4	1.8	0.7	17.8	1.5	0.6	26.6	27.9
18:1 ω9c	0.1	2.4	0.2	0.6	0.5	0.0	3.9	4.6
18:1 ω9t	0.2	5.3	0.1	0.1	0.3	0.0	6.0	7.4
18:0	0.1	0.6	0.9	1.7	0.0	1.6	5.0	5.5
Bacterial cy19:0	18.2	0.2	0.7	1.6	3.0	0.0	23.8	31.8
20:0	0.2	0.1	0.1	0.7	0.0	0.4	1.5	1.6
Total PLFA variance	29.3	17.0	3.8	25.4	5.7	4.3	85.6	100.0

in both responses. Third, ^{13}C NMR captures the bulk soil C forms and is not able to capture the subtle changes in C form in a small fraction of the soil organic matter that matters the most to microbial function. Moreover, not all drivers of microbial community structure and function are related to carbon. For example, we found that other macronutrients, N and P, were also very important in shaping the microbial communities (Ng et al., in review). Cusack et al. (2011) also found an interaction between mineral N deposition and the indigenous carbon reserves of two different soils, which caused a divergence in the microbial community structure and function after the N addition. In that case, N-addition to lowland forest soil with more labile carbon caused increases in bacterial dominance and hydrolytic enzyme activities, whilst N addition to highland forest soil with more stable C favoured fungal communities and oxidative enzyme activities (Cusack et al., 2011). Finally, the differences in the RDA and MRT results highlight the importance of interactions and/or associations among C forms leading to the observed patterns in soil microbial community composition and function; this further justifies the use of RDA and MRT together. Other studies on litter decomposition have found that there is a non-additive, synergistic mixture effect to decomposition rates when distinct litters decompose in a mix as opposed to in isolation (Hättenschwiler et al., 2011). One possible explanation for this is that greater heterogeneity of C forms also leads to greater interaction among the C forms. This warrants further investigation.

As the Cranbourne and Werribee soils have very different physicochemical properties, the patterns in microbial community composition and microbial activities here indicate a strong influence of the carbon composition of the OA. Both soils clearly showed that with the addition of 1% carbon to the soils, changes in their carbon composition occurs with changes in the microbial community composition and function 12 weeks after the application of OA. However, the magnitude of this effect is mediated by the soil physicochemical properties. In the sandy Cranbourne soil, Gw amendment resulted in the soil microbial composition being a lot more similar to its Werribee counterpart amended with the same OA than to its unamended soil. The same was not true for the amended Werribee soil, which is a clayey soil. The microbial composition in Gw amended Werribee soil reflects both the amendment and its unamended soil. However, it is worthy to note that the 1% carbon addition represented a bigger proportional increase in soil carbon for Cranbourne soil than Werribee soil. These differences in physicochemical properties of the soils may explain the variation in the treatments that were not explained by carbon forms. We would expect that over time, the relative importance of different environmental factors in determining structure and function of soil microbial community varies; such temporal factors are likely to be important, and therefore, deserve further attention.

5. Conclusions

The results of our study indicate that there is a very strong relationship between carbon composition, as determined by ^{13}C NMR analysis, soil microbial community composition and microbial activity. The addition of OA changes the soil C composition and alters the soil microbial community composition and its activity. Compost amended soils were most dissimilar from unamended soils for both microbial community composition and activity. A large proportion of these variations are explained by the relative content of alkyl-C, O-aryl-C, aryl-C and carbonyl-C. The differences in the patterns of soil microbial composition and microbial activity were associated with different C forms appearing as the most influential explanatory variable and the interaction between these C forms.

The results of this study demonstrate the direct relationship of soil carbon composition with soil microbial community structure and function. Such studies combining molecular approaches with functional measures will allow us to better assess effects of OA in agroecosystems and to examine SOM responses to management practices. Building on such a predictive approach to examine the relationship between carbon forms, microbial community structure and function will lead us towards not only understanding the relationship between the soil living and non-living components, but may enable us to predict outcomes for the use of organic amendments in the future based on the chemical composition of the applied organic matter.

Acknowledgement

This work was supported by funding from Monash Sustainability Institute Australia, and Brown Coal Innovation Australia. Experimental set-up, monitoring and chemical analysis was funded by the Department of Environment and Primary Industries (DEPI), Victoria. The green waste used in this study was kindly supplied by SITA Australia. Respiration data was collected by Dr Peter Fisler and Nick O'Halloran of DEPI. TRC also thanks Australian Research Council for financial support (FT12100463).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2013.12.004.

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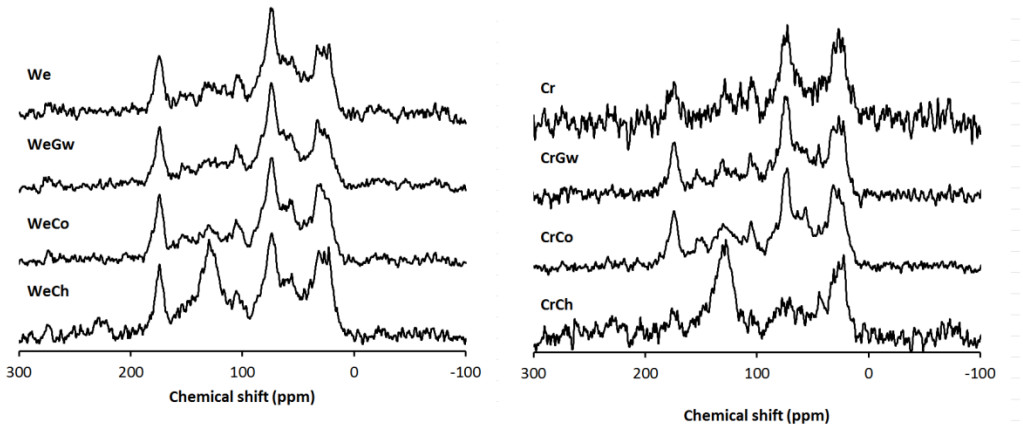
Please note that the following tables and figures referred only to Chapter 2.

Supplementary Table S1 Relative content of carbon forms in the organic amendments and samples. We = Werribee soil, Cr = Cranbourne soil, Gw = greenwaste, Co = composted greenwaste, Ch = charred greenwaste (e.g. CrGw refers to Cranbourne soil amended with greenwaste).

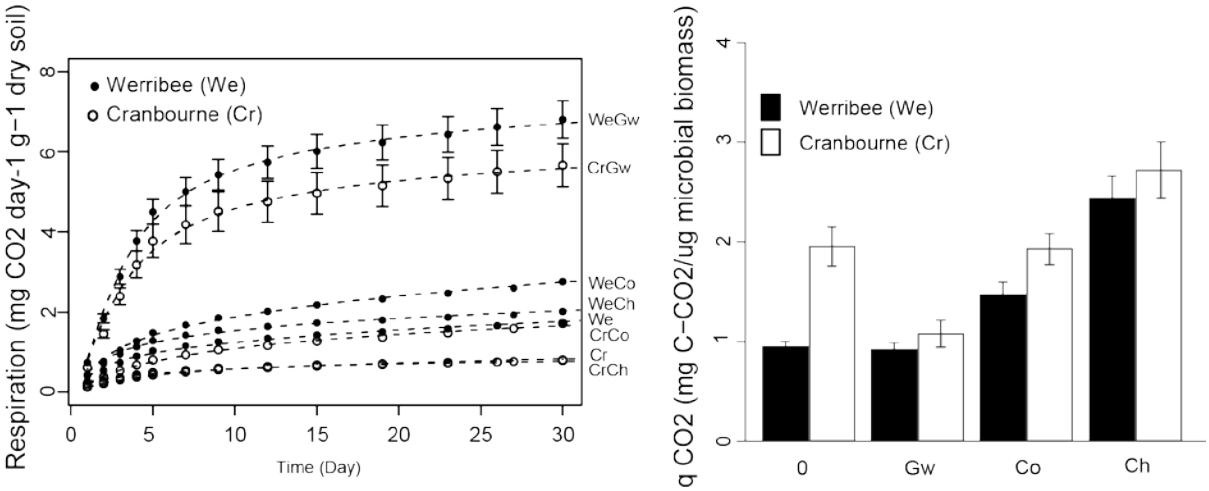
	Alkyl-C	N-alkyl-C	O-alkyl-C	Aryl-C	O-aryl-C	Carbonyl-C
We	20.6	9.3	33.6	15.6	6.5	14.5
Cr	28.0	7.1	30.9	16.7	4.2	13.2
WeGw	18.7	8.1	33.5	16.6	7.0	16.1
CrGw	21.1	7.6	32.5	16.6	8.1	14.1
WeCo	19.8	7.5	30.2	17.4	9.2	15.8
CrCo	21.3	8.3	30.0	20.2	7.4	12.9
WeCh	17.2	6.6	25.7	29.3	8.3	12.9
CrCh	17.6	2.7	15.2	39.8	11.8	12.9

Supplementary Table S2. Microbial activity (mean ± se). Units for PHOS, BGL, PPO, POX = μmols/ h/ g soil; PMN = μg NH_4^+/ g soil. We = Werribee soil, Cr = Cranbourne soil, Gw = greenwaste, Co = composted greenwaste, Ch = charred greenwaste (e.g. CrGw refers to Cranbourne soil amended with greenwaste).). BGL = β – glucosidase, PHOS = phosphatase, PMN = potentially mineralisable nitrogen, PPO = polyphenol oxidase, POX = peroxidase

	PHOS	PMN	BGL	PPO	POX
We	1.68 (0.28)	2.23 (0.80)	0.62 (0.26)	45.9 (1.2)	10.52 (0.55)
WeGw	2.47 (0.50)	22.21 (7.27)	0.52 (0.15)	46.7 (1.2)	11.43 (0.56)
WeCo	2.05 (0.16)	19.91 (5.71)	0.41 (0.08)	40.0 (1.1)	3.56 (0.28)
WeCh	1.29 (0.10)	3.57 (0.72)	0.13 (0.04)	35.1 (0.7)	3.63 (0.36)
Cr	0.01 (0.01)	0.09 (0.06)	0.00 (0.00)	39.5 (1.5)	6.92 (0.48)
CrGw	1.37 (0.16)	10.51 (1.71)	0.11 (0.07)	39.5 (0.2)	9.15 (0.16)
CrCo	0.22 (0.06)	3.59 (0.43)	0.00 (0.00)	35.3 (0.7)	1.61 (0.40)
CrCh	0.00 (0.00)	0.82 (0.20)	0.00 (0.00)	33.2 (0.8)	1.77 (0.22)



Supplementary Fig. S1 ^{13}C-NMR spectra of the samples. See methods for spectral assignments to carbon forms. We = Werribee soil, Cr = Cranbourne soil, Gw = greenwaste, Co = composted greenwaste, Ch = charred greenwaste (e.g. CrGw refers to Cranbourne soil amended with greenwaste).



Supplementary Fig. S2(A) Cumulative respiration (mean ± se) for the first 30 days of experiment (B) Metabolic quotient. Gw = green waste, Co = composted green waste, Ch = charred green waste. Note that total PLFA is used as microbial biomass for the calculation of qCO_2.

Preparation of organic amendments

The OA were prepared from one 20 m^3 batch of herbaceous green waste (i.e. leaves, grass clippings; approx. C:N 20:1). The green waste was derived from residential curbside waste collections in Melbourne and was received by us for processing on the same day of collection. The OA included fresh green waste (Gw), compost (Co) and char (Ch). Gw and Ch were derived from a 30-L subsample that was taken on the same day that the green waste was received. This subsample was dried for 48 h at 40 °C. After drying, the Gw portion (10 L) was stored at 22 °C in a sealed container. Char (Ch) was produced from the remaining 20 L by thermal treatment of the green waste at 450 °C in a pyrolysis batch reactor (Pacific Pyrolysis Pty Ltd, Gosford NSW, Australia). Compost (Co) was produced from the remainder of the batch by composting the green waste in a windrow of dimensions 1.2 m (high) by 3 m (width) by 8 m (length). The moisture content of the green waste was maintained at 50-60% (w/w wet basis) during composting. During the first month of composting, the windrow was turned twice-weekly with a loader and every 7 to 10 d thereafter. To determine when the compost was ready for use, the windrow was sampled every 7 to 14 days and tested using a commercially available compost maturity kit ('Solvita', Woods End Laboratory, Mt Vernon, ME) and a laboratory-based respirometry method (Sadaka *et al.* 2006). The compost was deemed ready for use with a Solvita score between 6 and 7 (Brewer & Sullivan 2003) and respiration of <2 mg CO_2-C g^{-1} VS day^{-1} (Thompson 2001). These criteria were met after 80 d composting. The Co sample was then stored at -18 °C until use. The Co sample was thawed for 48 h at 25 °C before applying to soil.

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Setup study design, carried out fieldwork and glasshouse work, performed most of the lab work, analysed all the data and wrote the manuscript.	90

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Antonio Patti	Involved in study design, writing manuscript	2
Timothy Cavagnaro	Involved in study design, writing manuscript	2
Michael Rose	Microbial biomass analysis, writing manuscript	2
Kevin Wilkinson	Prepared compost, writing manuscript	1
Cassandra Schefe	Involved in study design, writing manuscript	2
Ronald Smernik	^{13}C-NMR analysis and writing manuscript	1

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Chapter 3: Does organic input alter resistance and resilience of soil microbial community to drying?

This chapter has been submitted to a journal.

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Abstract

Grassland ecosystems in southeastern Australia are predicted to experience greater intensity and frequency of drying and rewetting events. In a terrestrial model ecosystem, we investigated the effects of compost on the resistance and resilience of the grassland soil ecosystem under drying and drying with rewetting events. Overall, compost addition led to improvement in soil moisture, greater plant available Colwell-P and higher plant $\delta^{15}N$. Soil C:nutrient ratios, mineral N content (NH_4^+ and NO_3^-) and soil microbial PLFA composition were similar between amended and unamended soils. Rainfall treatment led to differences in soil moisture, plant above-ground and below-ground biomass, plant $\delta^{15}N$, soil mineral N content (NH_4^+ and NO_3^-) and microbial biomass C, N and P composition but had no effects on soil C:nutrient ratios, plant available Colwell-P and soil microbial PLFA composition. There was little interaction between rainfall and compost amendment. The grassland soil

microbial community was generally resistant and resilient to fluctuations in rainfall regardless of compost amendment. These properties of the soil microbial community were translated to resilience but not resistance in soil functions. Overall, the results belowground showed much greater response to rainfall than compost amendment. This indicates that in this grassland, water is the main limiting factor for soil microbial community and nutrients are not strong co-limiting factors. Plant growth responses were sensitive to rainfall and compost amendment, but interaction effects were rare. Studies examining multiple global change variables acting in concert using the concept of resistance and resilience are of high policy relevance as they identify ways forward to sustainable management of productive ecosystems under global change.

Keywords: Soil enzymes; carbon cycling; 13-C NMR; PLFA; microbial activity

3.1.Introduction

Despite the fact that ecosystems are subject to multiple anthropogenically-driven environmental changes, our understanding of ecosystem responses to simultaneous global change drivers remains incomplete (Dukes *et al.*, 2005). There is a particularly pressing need for research on below-ground food web responses to concurrent global environmental change (Eisenhauer *et al.*, 2011). A previous study showed that a combination of atmospheric CO_2 enrichment, elevated nitrogen (N) and summer drought alter soil food webs and biodiversity through changes in soil moisture, below- and above-ground productivity, quality of litter inputs and rhizodeposition (Eisenhauer *et al.*, 2011). Such changes carry unknown implications for soil functions such as carbon and nutrient cycling, with equally unknown consequences for global biogeochemical cycling.

Southeastern Australia generally experiences high climatic variability. Grassland ecosystems form an important part of this landscape, where 350 million ha are grazed for

livestock and dairy production (ABS, 2013). Drought is a natural, periodic characteristic that shapes such landscapes, with the quantity and timing of rainfall influencing patterns of plant production (Dukes *et al.*, 2005) and carbon cycling (Chou *et al.*, 2008). In grassland ecosystems, soil microbial communities are generally considered resistant (i.e. they have greater ability to withstand disturbance) to climatic extremes as they experience regular, seasonal fluctuations in temperature and rainfall (Waldrop & Firestone, 2006). Soil microbial communities are also generally considered resilient (i.e. able to recover after disturbance), as they have been observed to recover from long dry periods quickly with rewetting events. These observations are based on microbial biomass, respiration and community-level physiological profiles (Bastida *et al.*, 2008, Griffiths *et al.*, 2003, Öhlinger, 1996). Nevertheless, rapid rewetting causes physiological stress to microbes (Kieft *et al.*, 1987, Lundquist *et al.*, 1999) and different microbial populations respond differently. Such differences in response lead to unique sets of active, prevailing soil microbial communities that may lead to alternative dynamics in ecosystem processes.

As climate projections suggest a future with increases in the frequency and severity of drought and extreme rainfall events (Alexander & Arblaster, 2009, Hennessy *et al.*, 2008), the ability of soil microbial communities to withstand or adapt to the changes remain unknown. While the grassland soil microbial community as a whole may appear to have great adaptability and plasticity, multiple stressors that are rapidly changing their habitat and resources may still alter the resistance and resilience of the soil community to subsequent disturbances (Calderón *et al.*, 2000, Steenwerth *et al.*, 2005). This may cascade into changes in ecosystem services from soil as the soil microbial community is a key regulator of soil processes through the transformation of soil organic matter.

Nutrient availability is also an important driver of soil ecosystem function and carbon cycling. Modern agriculture is heavily dependent on regular fertilizer inputs and this trend is

likely to continue in the coming decades, although some fertilizers, such as phosphorus and potassium, are derived from finite resources (Cordell *et al.*, 2009, Odegard & van der Voet, 2014, Vitousek *et al.*, 1997). The addition of organic amendments (OA) may offer an option to supplement/augment inorganic fertilisers and support sustainable, biologically regulated nutrient supply systems. Previous studies have shown that inputs of OA affect soil biota, plants and biogeochemical cycling (Bastida *et al.*, 2008, Ippolito *et al.*, 2010, Ryals & Silver, 2013). OA has been observed to improve primary productivity and net ecosystem C storage (Ryals *et al.*, 2014, Ryals & Silver, 2013). Microbial biomass and activity often increase with addition of OA (Bastida *et al.*, 2008) and improvements in soil organic matter with OA can persist for over a decade (Ippolito *et al.*, 2010).

Organic amendments are proposed to improve soil resilience to disturbance (Griffiths & Philippot, 2013). Organic matter amended soils have been observed to exhibit less pronounced changes in PLFA compared to unamended soils under drought conditions (Hueso *et al.*, 2012). Severe disturbances can lead to poor but stable and resistant states that require external inputs to provide a source of energy and nutrients to allow biological colonisation and improvement in soil functions (Ohsowski *et al.*, 2012). Besides energy and nutrients, OA may also improve soil structure, cation exchange capacity and water holding capacity, which combined with slow-release of nutrients may benefit below- and above-ground resilience to disturbance (Hargreaves *et al.*, 2008, Ryals *et al.*, 2014, Ryals & Silver, 2013). With these expected improvements to the soil on addition of compost, we expect that compost amendment would improve both the resistance and resilience of the soil microbial community, and therefore soil functions, to drying and rewetting cycles.

The objective of this study was to identify the responses of an intensively grazed grassland ecosystem to multiple disturbances in the form of variations in rainfall and organic amendment, particularly focusing on soil microbial community responses. To do so, we

determined (1) the above-ground and below-ground responses to drying and rewetting and, (2) examined if compost amendment alters the resistance and resilience of the soil microbial community to drying and rewetting cycles. Specifically, we examined the hypothesis that compost amendment improves the resistance and resilience of soil microbial community, and the processes of carbon (C), nitrogen (N) and phosphorus (P) that they govern.

3.2. Materials and Methods

Soil, Experimental Design and Sampling

In a terrestrial model ecosystem experiment, we collected intact soil cores from an intensively grazed grassland in the Toomuc Valley at Pakenham (38°0' S, 145°28' E). These intact soil cores (40cm length*15cm diameter) were then housed in temperature-regulated carts placed within a glasshouse (see Knacker *et al.*, 2004 for details of terrestrial model ecosystem approach). The field site was covered predominantly by ryegrass (*Lolium* sp.) and some ribwort plantain (*Plantago lanceolata*), carpet grass (*Axonopus affinis*) and finger grass (*Digitaria* sp.). The soil was a Brown Chromosol with 7.5% organic matter, C:N ratio of 11.1, $\delta^{13}C$ of -29.5‰, $\delta^{15}N$ of 3.9‰ and pH of 5.39 (H_2O). The green waste was collected from municipal green waste and composted following the method of Ng *et al.* (2014). Its characteristics were: total C (16.9%), total N (1.49%), total P (2440 mg/kg), $\delta^{13}C$ (-27.8‰); $\delta^{15}N$ (7.3‰), NO_3^- (485 mg/kg), NH_4^+ (30 mg/kg) and pH 8.36 (H_2O). It was applied on the surface at the rate of 30 t/ ha (based on dry mass), which was equivalent to 86 g (wet weight) per core.

Rain treatments were based on rainfall data from 1948 to 2012 for Pakenham from the Australian Bureau of Meteorology (2012) weather station at Scoresby, located 32 km to the northwest. The frequency of rain was determined by calculating the median number of rain

events and the number of days where the rainfall is greater than 1 mm, followed by random number generation using R 2.15.1. A rain event is defined by any precipitation in a day or over consecutive days. Accordingly, in this experiment, rain was applied once at each rain event for March and April and over two consecutive days for each rain event in May. For normal rain, which is the control rain treatment, we determined total amount of rain from the decile 5 (median) rainfall for each autumn month (March, April and May) over 1948 to 2012. This corresponds to 47.8 mm, 65.0 mm and 83.2 mm for March, April and May, respectively. For drought, we used the lowest rainfall recorded over the same period. This corresponds to 4.0 mm, 18.4 mm and 12.4 mm for March, April and May respectively. The heavy rainfall after drought on day 87 (150 mm in a day) was based on record high rainfalls in Victoria.

Cores were assigned in a randomised complete block design. Each full set of treatments was housed in a temperature-regulated cart and each treatment was replicated five times. The cores were equilibrated for 2 weeks and maintained under normal rain conditions using deionised water. The cores were organised into randomised blocks, housed within a controlled environment glasshouse. The photoperiod was 16 h day/ 8 h night. Day temperature was maintained at maximum 24 °C, 20 °C and 16 °C, respectively, for March, April and May. The cores were destructively sampled after 3 months. Samples were taken from the 0-5 cm depth and sieved to < 2 mm. Subsamples were kept at 4 °C for enzyme analysis or -20 °C for other analyses, followed by air drying of the remaining sample for chemical analysis.

Soil physical and chemical properties

Unamended and amended soil samples were analysed for a suite of chemical properties. A high-frequency induction furnace (LECO Pty Ltd) was used to measure total soil C and N. Total soil P was determined by method 17C1 in Rayment and Lyons (2011).

Mineral N was extracted with 2 M KCl (1:4 soil extractant) and measured colorimetrically following Forster (1995) and Miranda et al. (2001) for NH_4^+ and NO_3^-, respectively. Colwell P was extracted with 0.5 M $NaHCO_3$ (1:100 soil extractant) and measured following D'Angelo *et al.* (2001).

Plant Biomass

The above-ground biomass was obtained by cutting the grass to the soil surface. Roots were extracted by wet sieving using 1 mm and 0.25 mm sieves. Samples were dried at 40 °C for 3 days. Plant C, N, $\delta^{13}C$ and $\delta^{15}N$ content were determined on an ANCA GSL2 elemental analyser interfaced to a Hydra 20-22 continuous-flow isotope ratio mass spectrometer (Sercon Ltd., UK). Stable isotope data are expressed in the delta notation ($\delta^{13}C$ and $\delta^{15}N$), relative to the stable isotopic ratio of Vienna Pee Dee Belemnite standard (R_{VPDB}= 0.0111797) for C and atmospheric N_2 (R_{Air} = 0.0036765) for nitrogen.

Microbial community analyses

Soil microbial biomass C, N and P were determined by the chloroform fumigation-extraction technique as described by Vance et al (1987), but using 4 g of fresh soil for both fumigated and non-fumigated sub-samples. Microbial biomass C was quantified by dichromate digestion of fumigated and unfumigated samples as described by Cai *et al*. (2011). Microbial biomass N was determined using the method of Joergensen and Brookes (1990) to quantify ninhydrin-reactive N, and microbial biomass P was determined via the method of Jeanotte *et al*. (2004).

Soil microbial phospholipid fatty acid (PLFA) was extracted using a method modified from Bligh and Dyer (1959) using citrate buffer (Nielsen & Petersen 2000) and alkaline methanolysis of phospholipids (Bossio & Scow, 1998). The PLFA profile was identified using a Varian CP 38/00 gas chromatograph fitted with 5 % phenyl:95 % methylsiloxane column (Varian, Walnut Creek CA, USA). The fatty acids i15:0, a15:0, 15:0, i16:0, 16:1ω7, i17:0, a17:0, 17:0cy, 17:0, and 19:0cy were chosen as bacterial biomarkers and linoleic acid (18:2ω6,9) was chosen as the biomarker for decomposer fungi (see Frostegård & Bååth, 1996 and references therein).

Microbial activity

We assayed the activities of five enzymes in soil. β-glucosidase (BGL), phosphatase (PHOS) and polyphenol oxidase (PPO) activities were determined according to procedures modified from Allison and Jastrow (2006). We have found these assays to provide a good indication of soil microbial community activity in our earlier work on the impact of organic amendments to the soil (Ng *et al.*, 2014; see Chapter 1 and 2). Peroxidase (POX) was assayed using a method modified from Frey *et al.* (2000) and Johnsen and Jacobsen (2008). Urease (URE) was assayed following a method modified from Kandeler and Gerber (1988). 0.5 mL of homogenised soil slurry in sodium acetate buffer (pH 5, 50 mM; 5g in 50 mL) was combined with 0.5 mL of substrate solution made using the acetate buffer. BGL, PHOS, URE, PPO and POX were incubated for 2h, 2h, 5h, 1h and 10 mins, respectively. A background soil control and a substrate control were analysed for all enzymes. For URE, an additional 0.01 mL of toluene was added in all replicates and controls. At the end of incubation, NH_4^+ was extracted using 4 M KCl and NH_4^+ measured using the Forster (1995) method modified for a 96-well microplate.

Statistical Analysis

Randomised block design ANOVA was performed. Where assumptions of normality and homoscedasticity were not met, transformations were carried out and compared to results of untransformed data. Where similar statistical significance was obtained, results of the untransformed data were presented. Transformation was retained for the analysis of the following: log (n+1) transformation for aboveground biomass for May and $\delta^{15}N$, and rank transformation for BGL and PHOS. Post-hoc multiple comparisons were carried out using a least significant difference (LSD) test with *p*-values adjusted using Bonferroni.

Resistance, that is the capacity of the microbial community to resist change following a disturbance, was calculated as the difference between drying and control treatments relative to controls. Resilience, that is the capacity of the microbial community to recover following a perturbation, was calculated as the difference between rewetting and control treatments relative to controls. Compost-amended soils under normal rainfall were used as controls for amended samples and unamended soils under normal rainfall were used as controls for unamended samples. Randomised block design ANOVA was carried out as above on the actual response variables for all treatments to minimise introduced errors due to multiple one-way ANOVAs.

Multivariate analysis was carried out using standardised data. Microbial biomass and PLFA data were standardised using chord transformation. Kendall τau (τ) was used to examine correlation between fatty acids. Most fatty acids were correlated. Five fatty acids had < 50% correlation with other variables. Sequential addition of fatty acids to the analysis did not add meaningful patterns to the ordination. Therefore, these five fatty acids were sufficient for the final nonmetric multidimensional scaling (NMDS). All results were checked by using chi-square transformation, cluster analysis and principal component analysis (PCA).

Cluster analysis was done using Ward's hierarchical clustering on the Bray-Curtis dissimilarity matrix. The results were generally the same and only NMDS and cluster analysis are shown.

Data analysis was carried out on R 2.15.1 (R Core Team 2012) using the alr3 package (Weisberg, 2005) and the agricolae package (Mendiburu, 2012) for randomised block ANOVA and LSD test, respectively. Ordinations were carried out using the *vegan* package (Oksanen *et al.*, 2012).

3.3. Results

Soil physical and chemical properties

Soil moisture differed across the different rainfall treatments applied (Fig. 3.1; $F_{2,22}$ = 204.1, p <0.001). Soil moisture was highest in rewetting, followed by normal rainfall and drying treatments. Additionally, amended soils had consistently higher soil moisture than unamended soils ($F_{1,22}$ = 10.4, p = 0.039). Soil C:N, C:P and N:P ratios were similar across treatments (Fig. 3.1). Mineral N content (NH_4^+ and NO_3^-) was different only between rain treatments (Fig. 3.1; $F_{2,22}$ = 11.4, p <0.001 for NH_4^+; $F_{2,22}$ = 53.9, p <0.001 for NO_3^-). Soil NH_4^+ and NO_3^- were higher in the drying and rewetting treatments compared to normal rain. Plant available (Colwell) P was only different between amended and unamended treatments ($F_{1,22}$ = 6.4, p = 0.019). Compost amended soils had higher Colwell-P compared to unamended soils.

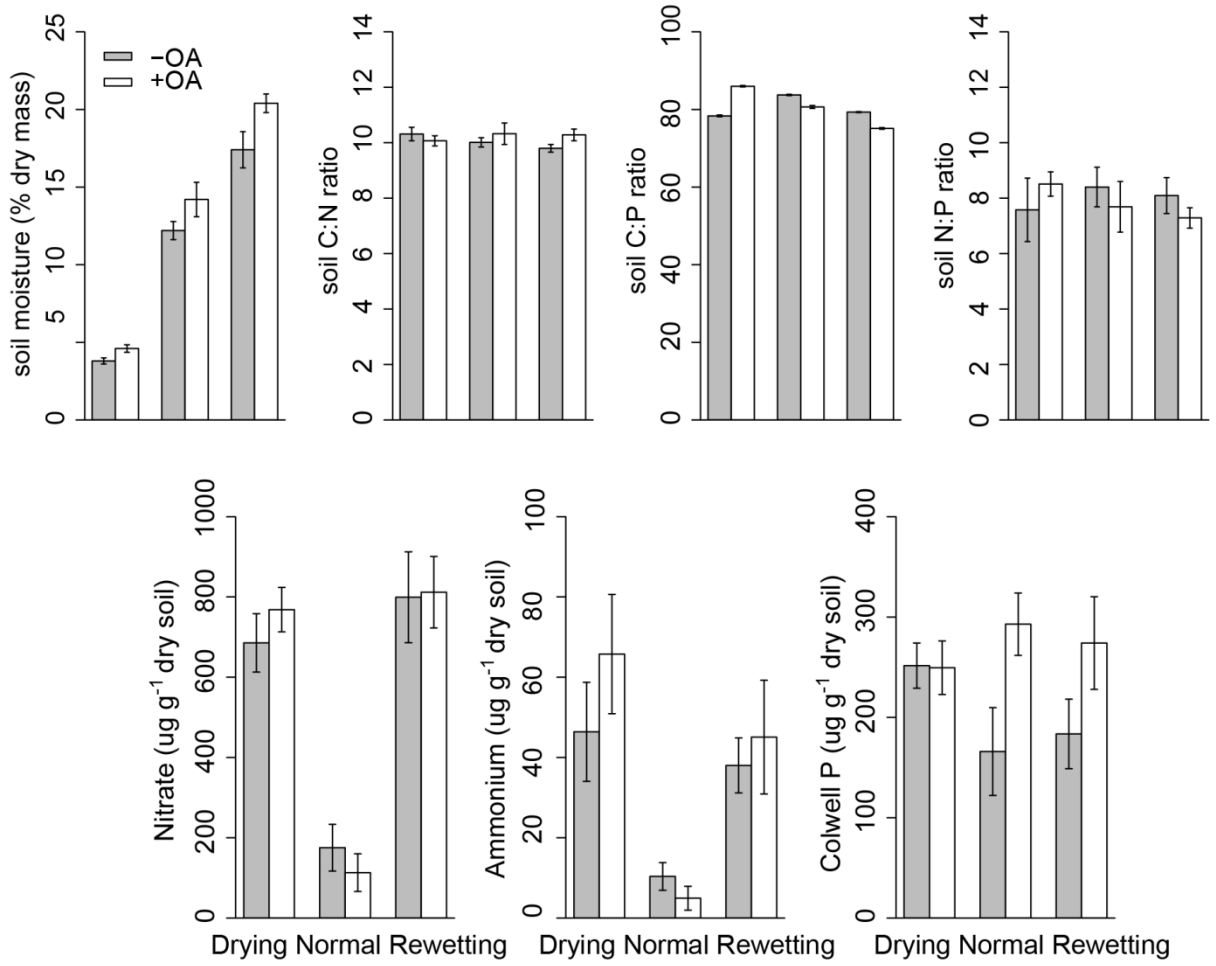

Fig. 3.1 Chemical and moisture properties of soil with OA treatment (mean ± standard error) under different rainfall. All bar plots are similarly grouped from left to right for drying, normal and rewetting treatments. –OA = unamended soils; +OA = amended soils.

Plant biomass and elemental content

Plant biomass was mainly affected by rain treatment (Fig. 3.2; $F_{2,22} = 37.3$, $p < 0.001$ in April; $F_{2,22} = 65.3$, $p < 0.001$ in May; $F_{2,22} = 8.2$, $p = 0.002$ for roots), where above-ground biomass was higher under normal rain in the second and third months of the experiment. Root biomass was also higher under normal rain compared to drying, but similar when compared to rewetting. The addition of OA also affected above-ground biomass at the end of the

experiment (May) ($F_{1,22}$ = 12.1, p = 0.002); specifically, above-ground biomass was lower in compost amended than unamended soils.

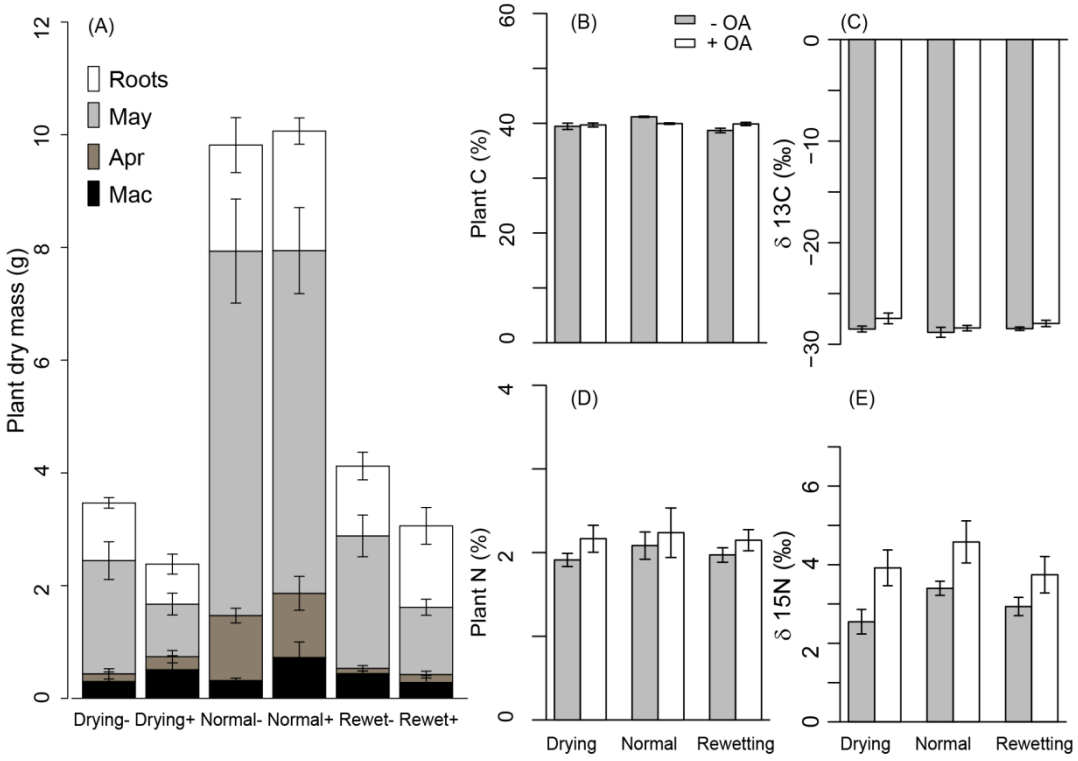

Fig. 3.2(A) Plant aboveground in March, April and May, and root biomass in May; where (+) = compost amended soils, (-) = unamended soils. (B,D) Plant C and N contents and, (C,E) Plant $\delta^{13}C$ and $\delta^{15}N$ in May.

Plant aboveground C content was affected by OA only under normal and rewetting (Fig 3.2B; $F_{2,22}$ = 5.5, p = 0.013). Under normal rain, there was higher plant C in unamended compared to compost amended soil but the reverse was found in the rewetting treatment, i.e. plant biomass C was higher in compost amended soil compared to unamended soil. Plant $\delta^{13}C$ varied over a narrow range between -27 and -29 ‰ (Fig. 3.2C), and this indicates that the plants were not drought stressed. Plant aboveground N content was similar across all treatments (Fig. 3.2D). However, their $\delta^{15}N$ value was affected by rain treatment ($F_{2,22}$ = 3.6,

p = 0.04) and addition of OA (Fig. 3.2E; $F_{1,22}$ = 18.5, p <0.001). Specifically, plant $\delta^{15}N$ was higher with addition of OA; this is consistent with the OA having a higher $\delta^{15}N$ than the soil. Plant $\delta^{15}N$ was also more similar between drying and rewetting treatments compared to normal rainfall.

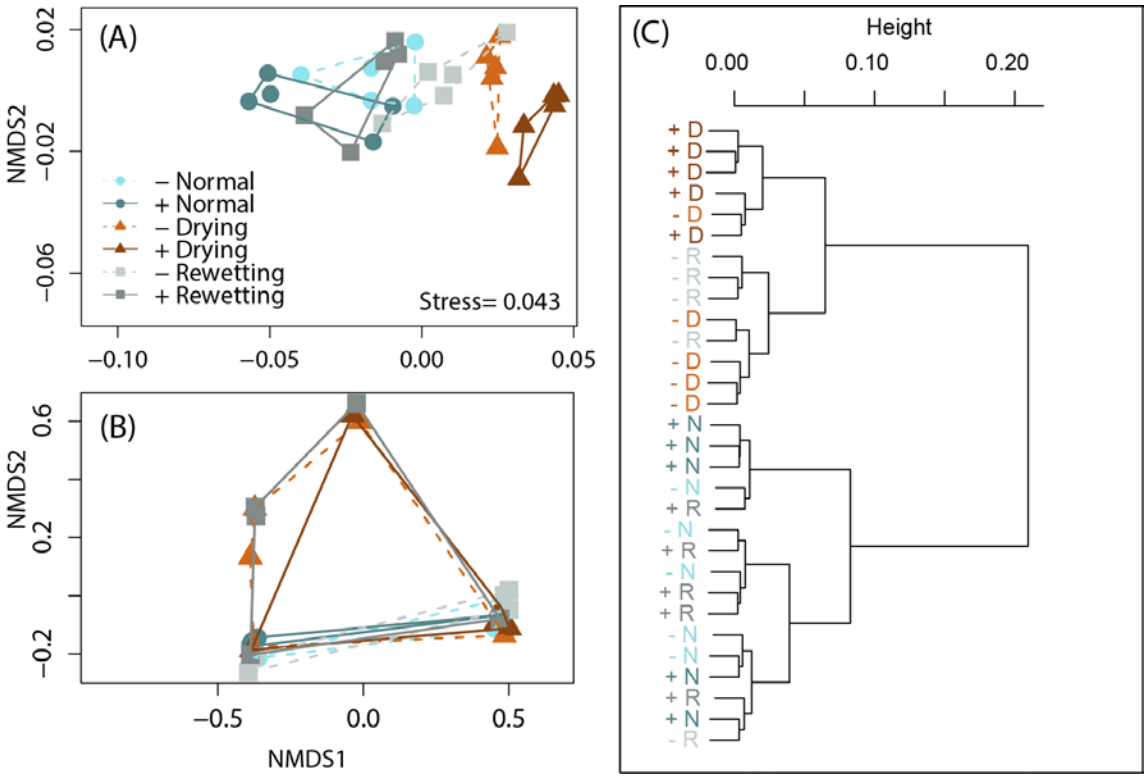

Fig. 3.3 Variations in soil microbial composition with amendment treatment under different rainfall regimes. Nonmetric multidimensional scaling (NMDS) plots of (A) microbial biomass C, N and P composition and (B) microbial PLFA composition. Distances among points express relative dissimilarities in microbial composition. (C) Cluster analysis of microbial biomass C,N and P composition indicates two main groups, one group consisting of all soils which experienced normal rainfall and amended soils that experienced rewetting and a second group consisting of all soils which experienced drying and unamended soils that experienced rewetting.

Soil microbial composition and structure

Soil microbial biomass C, N and P composition indicated their elemental compositions under normal and rewetting treatments were more similar than for the drying treatment (Fig. 3.3). Additionally, cluster analysis indicated that soil microbial biomass C, N and P composition was more similar between amended soil under rewetting and soils under normal rain. Soil microbial PLFA composition showed no clear pattern with regards to the treatments.

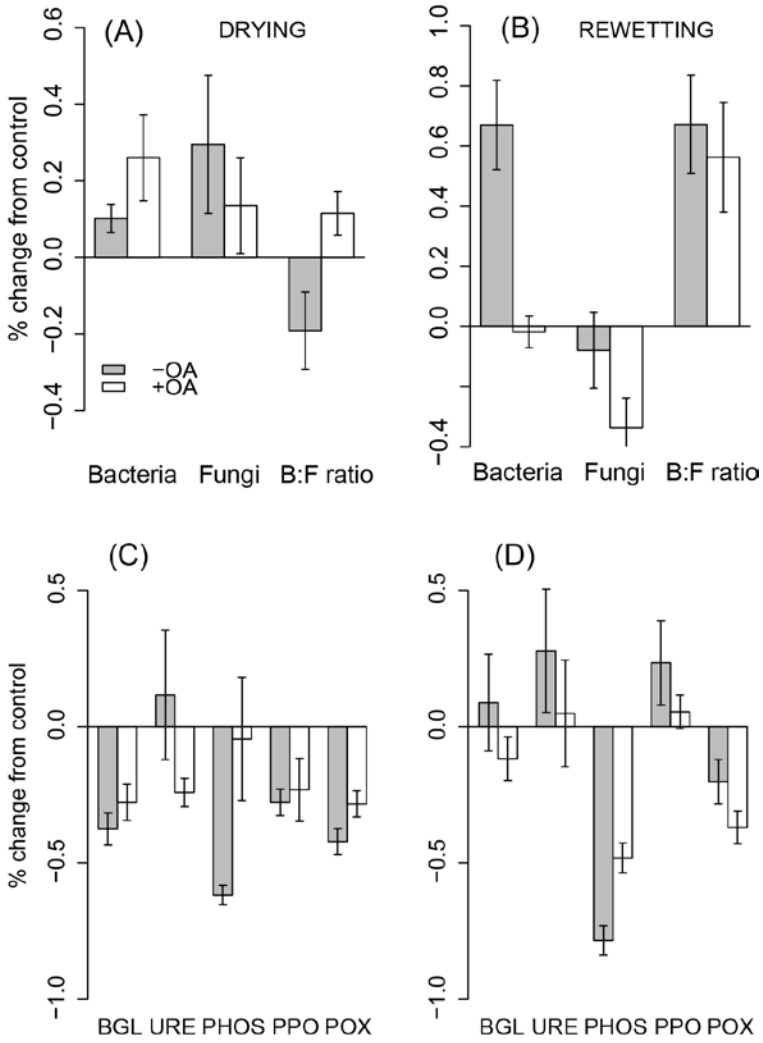

Fig. 3.4 Effects of OA on resistance and resilience of soil microbial PLFA composition and soil functions to drying and rewetting. (A,C) Resistance of microbial composition and microbial activity to drying, (B,D) resilience of microbial composition and microbial activity following rewetting. –OA = unamended soils; +OA = amended soils.

To assess the resistance of the soil microbial communities (i.e. their ability to withstand disturbance), we calculated the difference in soil microbial structure between drying and control relative to the control using bacterial biomass, fungal biomass and bacteria-to-fungal ratio (B:F ratio). Therefore, the resistance of microbial community structure is examined with respect to controls (Fig 3.4A). OA altered the resistance of bacterial biomass to drying ($F_{2,22} = 15.7$, $p < 0.001$). Bacterial biomass was resistant to drying without organic amendment and the addition of compost further increased the bacterial biomass even with drying. Fungal biomass was resistant to drying regardless of OA treatment ($F_{1,22} = 0.8$, $p = 0.371$). The B:F ratio was similarly resistant to drying regardless of OA treatment ($F_{1,22} = 2.2$, $p = 0.152$).

To assess the resilience of the microbial communities (i.e. their ability to recover after disturbance), we calculated the difference between the rewetting and control relative to the control (Fig. 3.4B). With rewetting, the resilience of bacteria to drying differed between amended and unamended soil ($F_{2,22} = 15.7$, $p < 0.001$). With the addition of OA, bacterial biomass after rewetting was similar to that of normal rain levels. The addition of OA did not alter the resilience of fungal biomass or B:F ratio. Fungal biomass under the rewetting regime was similar to that of normal rainfall regardless of the addition of OA ($F_{1,22} = 0.8$, $p = 0.371$). The B:F ratio was higher in the rewetting compared to the drying treatment regardless of OA treatment ($F_{2,22} = 13.8$, $p < 0.001$).

Microbial activities

Similar to the microbial community composition and structural responses, resistance of microbial activities, as indicators of soil functions, was calculated as the difference of soil function between drying and control relative to the control. Therefore, the resistance of soil

functions was examined with respect to controls. BGL, PHOS, PPO and POX activities had low resistance to drying (Fig. 3.4C; $F_{2,22} = 4.2$, $p = 0.029$; $F_{2,22} = 9.5$, $p = 0.001$; $F_{2,22} = 7.5$, $p = 0.003$; $F_{2,20} = 35.4$, $p < 0.001$). This was indicated by the observed lower than control microbial activity with drying. The addition of OA did not alter the resistance of BGL, PPO or POX to drying but improved PHOS resistance to drying. URE was unaffected by drying regardless of the amendment treatment ($F_{2,22} = 0.9$, $p = 0.406$).

As above, resilience of the soil functions was calculated as the difference between the rewetting and control treatments relative to the control treatments (Fig. 3.4D). BGL was resilient to drying with or without OA ($F_{2,22} = 4.2$, $p = 0.029$). PPO was similarly resilient to drying with or without OA ($F_{2,22} = 7.5$, $p = 0.003$). Both BGL and PPO returned to control levels with rewetting in both amended and unamended soils. PHOS resilience was low but the addition of OA improved PHOS resilience to drying ($F_{2,22} = 9.5$, $p = 0.001$). URE was unaffected by rewetting regardless of the amendment treatment ($F_{2,22} = 0.9$, $p = 0.406$). Of the soil functions studied here, OA altered only the resilience of POX ($F_{2,20} = 5.9$, $p = 0.009$). The addition of OA reduced the resilience of POX to drying.

3.4. Discussion

Overall, compost addition led to an increase in soil moisture, greater plant available Colwell-P and higher plant $\delta^{15}N$. Soil C:nutrient ratios, mineral N content (NH_4^+ and NO_3^-), soil microbial PLFA composition were similar between amended and unamended soils. Rainfall treatment led to differences in soil moisture, plant above-ground and below-ground biomass, plant $\delta^{15}N$ content, mineral N content (NH_4^+ and NO_3^-) and microbial biomass C, N and P composition but had no effects on soil C:nutrient ratios, plant available (Colwell) P and soil microbial PLFA composition. There was little interaction between rainfall and compost

amendment, which was found to affect only plant above-ground C content, bacterial biomass, POX activity and microbial biomass C, N and P composition. We expected that compost amendment would improve the resistance and resilience of the soil microbial community to drying and rewetting cycles. Bacterial biomass was resistant to drying and further increased with compost despite drying, but the resistance and resilience of fungal biomass and B:F ratio to drying were unaffected by compost addition. Compost addition also did not alter the resistance and resilience of most soil functions to drying and rewetting.

We found few interaction effects of compost addition and rainfall treatments for soil microbial community composition and structure. Cluster analysis of soil microbial biomass C, N and P composition indicated a possible interaction effect, where the analysis indicated two main groups. One group consisted of compost amended soil under rewetting, and both amended and unamended soils under normal rainfall. The other group consisted of unamended soil under rewetting, and both amended and unamended soils under drying. Given these differences in microbial biomass elemental composition between amended and unamended soil under rewetting occurred while their microbial PLFA composition and B:F ratio remained similar, this may indicate that the compost amendment altered soil microbial behaviour and physiology (Blagodatskaya *et al.*, 2007, Manzoni *et al.*, 2010). Prior studies have also found physiological responses to rewetting without changes in microbial community composition (Griffiths *et al.*, 2003).

One prior study reported that a fungal-based food web of grassland soil was resistant but not resilient to drying (De Vries *et al.*, 2012). In our study, fungal biomass was resistant and resilient to drying regardless of compost addition. Bacterial biomass was also resistant to drying without compost addition and addition of compost increased bacterial biomass despite drying conditions. Interestingly, bacterial biomass in the compost amended soils returned to control levels with rewetting but unamended soil had a significantly higher bacterial biomass.

It is unclear what could have caused this response and this is worthy of further investigation. The low B:F ratio under drying and the high B:F ratio after rewetting indicated a rapid change in microbial structure. Fungi can be resistant to drying and are less sensitive to changes in moisture although they tend to decrease when the soil becomes saturated (Drenovsky *et al.*, 2004, Unger *et al.*, 2009, Yuste *et al.*, 2011). We did not observe water saturation in our soils but we did observe lower fungal biomass with rewetting compared to soils under drying conditions. In a mild rewetting study using two soils, an inconsistent response from fungal biomass was observed whereby one soil had higher fungal biomass with rewetting while the other did not respond to rewetting (Steenwerth *et al.*, 2005). Changes in other physicochemical properties of the environment and interactions of microbes with other soil biota have been proposed to be at play. Furthermore, we measured these changes less than three days after the rewetting and soil moisture remained higher in the rewetting treatment, so these differences in microbial structure may merely reflect the temporal dynamics in the soil physicochemical environment.

We expected soil microbial activity to be more responsive to drying and rewetting compared to soil microbial composition or structure. Indeed we observed that soil functions changed with rainfall regimes, with a general decline in all enzyme activities under drying. However, microbial activity returned rapidly to that of control levels with rewetting, indicating soil functional resilience. Upon rewetting, rapid resuscitation of the soil microbial community, together with an immediate increase in activity, maximises the temporal pulse in resource availability (Dijkstra *et al.*, 2012, Placella *et al.*, 2012). The increases in microbial activity, however, did not alter the available N content in soils that experienced rewetting, which were similar in both amended and unamended soils, and similar to that of soils which experienced drying. As URE activity was unaffected by rainfall or OA, N was unlikely to be limiting in this system.

As microbial activity is sensitive to drying, we expected that the addition of OA may improve resistance and resilience of soil functions to drying. However, we found that BGL, PHOS and PPO were only affected by rainfall. The only interaction effect observed for soil functions was POX activity. The addition of OA did not improve the resistance of POX to drying but it reduced POX resilience to drying. POX activity has been reported to decrease with drying and this decline has been associated with reductions in fungal biomass and species richness but these observations did not extend beyond a drought period (Toberman *et al.*, 2008). Besides fungi, actinomycetes are central to the production of phenol oxidase and peroxidases for the degradation of phenolic compounds indicative of more recalcitrant organic matter (Kirk & Farrell, 1987, Sinsabaugh, 2010). As we observed fungal biomass was similar between amended soil that experienced rewetting and its normal rainfall control, actinomycetes may have an important role in our ecosystem. It is unclear why POX resilience to drying should be reduced with compost addition. It could be a simple case of the soil microbes preferentially exploiting the sudden pulse of labile resources from compost. Measures of POX over a longer period post-rewetting would be necessary to test this possibility.

The similarity of the overall microbial PLFA composition across compost amendment and rainfall variations, the general resistance and resilience of microbial structure and resilience of soil functions in this grassland can be attributed to various factors. First, the chemical nature of the compost is an important determinant of its decomposition and transformation (Fontaine *et al.*, 2011, Kallenbach & Grandy, 2011, Pascault *et al.*, 2013). For example, prior study has shown that the carbon composition of the OA strongly influences microbial PLFA composition and activity through changes in soil carbon composition (Ng *et al.*, 2014; see Chapter 2). The carbon composition of the soil organic matter influences its chemical, physical and biological interaction within the soil matrix and determines its

stability and accessibility to the soil microbial community (Kögel-Knabner *et al.*, 2008). We found that the soil carbon composition, as determined by ^{13}C-solid state NMR, was relatively similar across all our treatments (see supplementary Fig S3.1). As compost is a form of stabilised organic matter, its chemical nature makes it accessible only to a subset of the soil microbial community (Fontaine *et al.*, 2003, Pascault *et al.*, 2010). As such, depending on the environment and therefore organo-mineral interactions, the decomposition of compost may be slow. In one field study, it has been observed that only 12% of compost was decomposed 3 years after amendment (Ryals & Silver, 2013). This slow rate of decomposition means that hysteresis may have an important role that cannot be addressed within the time scale of this study.

Second, previous studies have reported that soil microbial communities inhabiting environments that regularly observe great fluctuations in environmental conditions are relatively resistant and resilient to such fluctuations in their environment (Fierer *et al.*, 2003, Griffiths *et al.*, 2003). This in part is attributed to physiological properties of the soil inhabitants and in part to permanent changes in the physical domain of the soil with such fluctuations. For example, the collapse of macro- and meso-pores in soil or organic matter aggregates when hydrogen-bonded water is lost is irreversible with rewetting. These can explain the similarity in responses from soil microbial communities of different farming systems in California's hot, largely rain-free summer to soil drying and re-wetting (Lundquist *et al.*, 1999). Combined with a history of intensive land use, a soil microbial community that is resistant and resilient to disturbances may have evolved earlier to inhabit this habitat (Ge *et al.*, 2008, Martiny *et al.*, 2006, Ohsowski *et al.*, 2012). A comparison of microbial communities across eight land use across California has found that agricultural management had larger effects on microbial composition than elevation or precipitation regime (Drenovsky *et al.*, 2010). Furthermore, the biotic legacy and site history also influences soil

physicochemical properties which have important effects on microbial community composition and physiology (Griffiths & Philippot, 2013). As our study site is an intensively grazed grassland with moderate nutrient availability, this may explain the lack of microbial activity response to compost amendment.

Third, the timing of rainfall, rather than quantity has been proposed to be critical to grassland community response (Chou *et al.*, 2008, Cruz-Martínez *et al.*, 2009, Weltzin *et al.*, 2003, Zeglin *et al.*, 2013). Following 5 years of rainfall addition in a grassland experiment, soil bacteria and archaea were found to be relatively similar to those found under ambient rainfall conditions (Cruz-Martínez *et al.*, 2009). The changes that were subsequently observed during the sixth and seventh year occurred only when additional rainfall exacerbated or alleviated periods of aberrant conditions in the ambient climate. When rainfall occurs over a cold winter, lower evaporation rates translate to increased soil water, less oxygen and translocation of oxides of iron and aluminium leading to lower soil redox potential (Berhe *et al.*, 2012). When rainfall occurs in a warmer spring, an increase in plant growth leads to higher evaporation rates and soil redox potential (Berhe *et al.*, 2012). These changes in soil chemical properties affect soil organic matter stabilisation, and may help to explain some of the responses seen in the microbial communities that rely on soil organic matter to support their activity for cell growth and maintenance.

The addition of compost did not improve the resistance of plant growth responses to drying. Both plant above-ground and below-ground productivity were negatively affected by drying. Despite the differences in availability of mineral N and indications from $\delta^{15}N$ that the plants did utilise the N from compost input, this was not translated to higher plant N in compost amended soils, although there seemed to be a trend of higher plant N with compost amendment. In fact, the above-ground N content of plants indicated that they were marginally deficient despite compost amendment. We do not discuss plant resilience here as this study

focuses on soil microbial community, and therefore, we have measured rewetting responses less than three days post-wetting. This duration is insufficient for examining plant resilience at the whole plot level.

3.5. Conclusion

In this study, we found that the grassland soil microbial communities are generally resistant and resilient to fluctuations in rainfall regardless of compost amendment. These properties of the soil microbial community were translated to resilience but not resistance in soil functions. Overall, the results below-ground showed much greater response to rainfall than compost amendment. This indicates that in this grassland, water is the main limiting factor for the soil microbial community, and nutrients are not strong co-limiting factors.

It has been observed that grassland soil microbial community robustness to rainfall alteration is not seen in above-ground macro-organisms (Cruz-Martínez *et al.*, 2009). In our study, we observed that plant growth was sensitive to rainfall and compost amendment, but interaction effects were rare, or at least not discernible. Differences in above- and below-ground responses may lead to decoupling between below- and above-ground dynamics and affect biogeochemical cycling (Cruz-Martínez *et al.*, 2009), although others have found that microbial and plant processes can be synchronised following a water pulse (Dijkstra *et al.*, 2012). Given there may be a time lag and longer duration in plant-microbial feedback that cannot be captured in our study, such interpretations remain to be confirmed by future studies. This study represents one of the few studies examining interactions between organic matter amendment and environmental change. Such studies, particularly if longer-termed, will allow us to identify climate-management-plant-soil microbial interactions and identify ways forward to sustainable management of productive ecosystems under global change.

3.6. Acknowledgement

This work was supported by funding from the Monash Sustainability Institute Australia, Brown Coal Innovation Australia and the Australian Research Council (ARC). NEL wishes to thank Pablo Galaviz, Mattia Pierangelini, Alicia Brown, Mani Shresta for assisting field and lab work. TRC also thanks the ARC for the award of a Future Fellowship (FT120100463).

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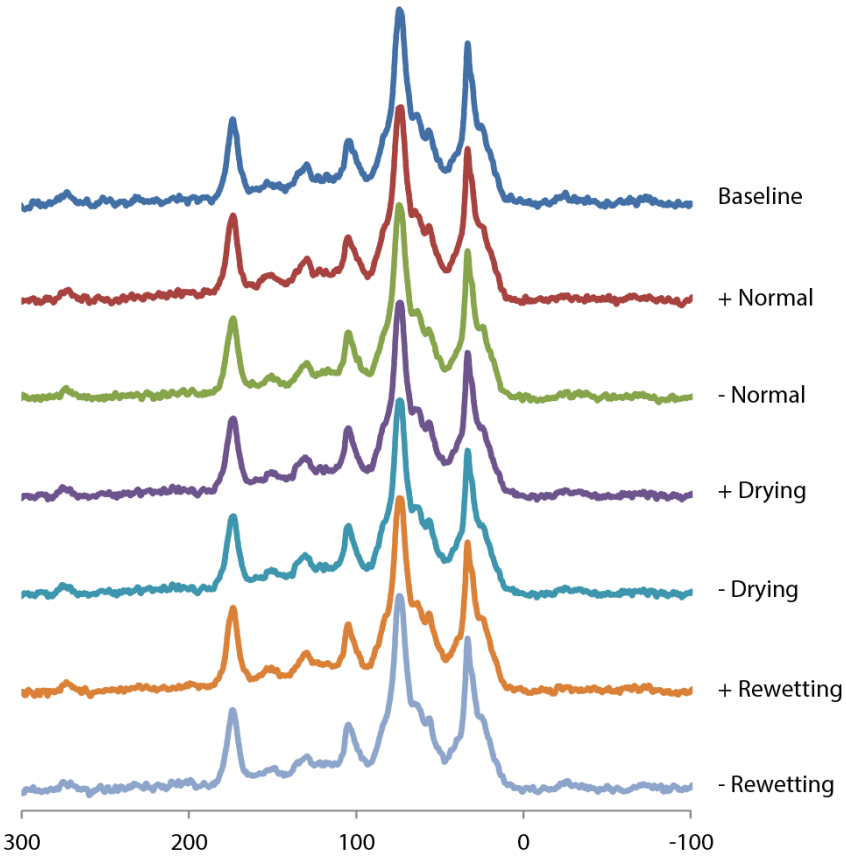
3.8. Supplements



Supplementary fig. S3.1 ^{13}C- solid state NMR spectra of the samples. See methods for spectral assignments to carbon forms in Ng *et al.* (2014). (-) refers to soil without compost amendment; (+) refers to soil receiving compost amendment.

Chapter 4: Where the elements go when it rains? Carbon and nutrient stocks of soil amended with stabilised organic matter.

Abstract

The addition of organic amendment to the soil has been proposed to improve soil organic matter in grasslands but little is known of the fate of organic matter added to grasslands, and even less is known about its fate under different rainfall regimes. This study quantified the carbon (C), nitrogen (N) and phosphorus (P) stocks after drying and rewetting events in an intensively grazed grassland ecosystem amended with compost at 3 depths (0 - 5 cm, 5 - 10 cm, 10 - 30 cm). Despite large variations in rainfall and the intensity of the rewetting event, we found that compost consistently increased C and N stocks at 5 to 10 cm and P stocks at 0 to 5 cm. C, N and P stocks at 10 to 30 cm were unaffected. It was concluded that compost amendment increased soil C, N and P stocks regardless of rainfall regimes but these subtle improvements can only be detected by careful consideration of soil depths.

4.1. Introduction

Grasslands cover approximately 40 % of terrestrial surface and stores a third of terrestrial carbon (C), of which considerably more is stored in soils than vegetation (White et al., 2000). However, overgrazing, woody encroachment and climate change have contributed to the degradation of grasslands (Asner et al., 2004; Bai et al., 2008). Besides representing a large reservoir of organic C, soil organic matter (SOM) is important as a reservoir of plant nutrients, soil moisture retention and soil aggregation.

Organic matter addition has been proposed as a means of replenishing soil organic matter in grasslands (Jackson et al., 2004; Powlson et al., 2012). The addition of organic amendments to the soil allows nutrient recycling and may divert wastes from landfills. Furthermore, organic amendments act as slow release fertilisers (Ryals and Silver, 2013), which is important for sustainable nutrient recycling as excess amounts of macronutrient leads to over-mining of micronutrients (Jones et al., 2013; Sheldrick et al., 2002). For the purpose of slow release, the addition of stabilised organic amendments, for example via composting, is preferred. Studies on grasslands have indicated that addition of stabilised organic amendment can lead to improvement in net primary productivity, SOM and available nutrients lasting years to decades (Ippolito et al., 2010; Ryals and Silver, 2013; Slavich et al., 2013).

As it stands, the fate of organic matter added to grasslands remains unclear (Ryals et al., 2014). One of the most important determinants of the fate of added organic amendments is climatic variables, such as rainfall. Rainfall regulates SOM turnover through its influence on primary productivity and soil heterotrophic activity, which occur through soil water and nutrient availability, temperature and pH (Borken and Matzner, 2009; Clark et al., 2009; Elhottová et al., 2006). As current climate projections suggests that the southeastern region of Australia will experience a future with more frequent and severe droughts and extreme rainfall events (Alexander and Arblaster, 2009; Hennessy et al., 2008), it is crucial to examine the fate of added organic amendment under the different rainfall regimes. As such, the objective of this study was to examine the carbon, nitrogen and phosphorus stocks after drying and rewetting events in an intensively grazed grassland ecosystem that received compost amendment.

4.2. Materials and Methods

Soil, Experimental Design and Sampling

Intact soil cores (15cm * 40cm) were collected from an intensively grazed grassland at Pakenham (Latttitude: -38.010551; Longitude: 145.472542) on the flooplain of the Toomuc Valley creek. The dominant plant was ryegrass (*Lolium* sp.) and also present were ribwort plantain (*Plantago lanceolata*), carpet grass (*Axonopus affinis*) and finger grass (*Digitaria* sp.). The soil was a Brown Chromosol with a pH 5.39 (H_2O), a C:N ratio of 11.1 and contains 7.5 % organic matter. The green waste was collected from municipal green waste and composted following Ng *et al.* (2014; see Chapter 2). It has a pH of 8.36 (H_2O) and was composed of 16.9 % C, 1.49 % N and 2440 mg/kg P. It has 485 mg/kg of NO_3^- (485 mg/kg) and 30 mg/kg of NH_4^+. The compost was applied onto the surface of each soil core at the rate of 30 ton/ ha (based on dry mass), which was equivalent to 86 g (wet weight/ core).

Details of the simulated rain treatments are found in Chapter 3. In short, each core under normal rain received 47.8 mm, 65.0 mm and 83.2 mm of simulated rainfall for March, April and May respectively. Each core under drying and rewetting received 4.0 mm, 18.4 mm and 12.4 mm for March, April and May respectively. Rain was applied once for each rain event for March and April and over two consecutive days for each rain event in May. The rewetting treatment received 150 mm of rainfall after drying on day 87, i.e. three days before the end of the experiment.

Cores were assigned according to a randomised complete block design. Each treatment was replicated five times. The cores were equilibrated for 2 weeks and maintained under normal rainfall with deionised water. They were organised into randomised blocks, each housed within temperature-regulated carts placed within a controlled environment glasshouse (see Knacker et al., 2004 for details of terrestrial model ecosystem approach). The photoperiod was 16 h day/ 8 h night. Day temperature was maintained at maximum 24 °C, 20

°C and 16 °C respective for March, April and May. The cores were destructively sampled at 0-5 cm, 5-10 cm and 10-30 cm soil depths, 3 months after the addition of the compost. For all depths, samples were sieved to < 0.2 cm, air dried and ground for elemental analysis. For soils at 0 to 5 cm, available N (NH_4^+, NO_3^-) and P (Colwell-P) were also measured using fresh and air dried samples, respectively.

Elemental analysis of soils and compost

Total soil C and N elemental analysis were performed using a Vario Microcube instrument operated in C,H,N mode. Total soil P was determined by method 17C1 in Rayment and Lyons (2011). Mineral N was extracted with 2 M KCl (1:4 soil extractant) and measured colorimetrically following Forster (1995) and Miranda et al. (2001) for NH_4^+ and NO_3^- respectively. Colwell P was extracted with 0.5 M $NaHCO_3$ (1:100 soil extractant) and measured colorimetrically following D'Angelo et al.(2001).

Statistical Analysis

Randomised block design ANOVA was performed. Where assumptions of normality and homoscedasticity are not met, transformations are carried out. These results were compared to results of untransformed data. As they are the same, results of the untransformed data are shown. Post-hoc multiple comparisons were carried out using least significant difference (LSD) test with *p*-values adjusted using bonferroni. Data analysis was carried out on R 2.15.1 (R Core Team 2012) using agricolae package (Mendiburu, 2012) for LSD test. Ordinations were carried out using vegan package (Oksanen et al., 2012).

4.3. Results

Carbon stocks were altered with compost amendment only at 5 to 10 cm depth in the profile (Fig. 4.1; $F_{1,22} = 6.7$, $p = 0.017$). Specifically, C stocks were higher in amended soils (mean ± standard error = 2.8 ± 0.1 kg/m^2) compared to unamended soils (2.5 ± 0.2 kg/m^2) in the 5 to 10 cm soil layer, but not the other soil layers. Rainfall treatment did not have any significant effects on the C stocks nor did it alter the effect of compost on C stock at all depths. Cumulative C stock from 0 to 30 cm was similar across OA and rain treatments.

Nitrogen stock was also altered with compost amendment at 5 to 10 cm (Fig. 4.1; $F_{1,22} = 5.4$, $p = 0.030$). Soil N stocks were higher in amended soils compared to unamended soils. Rainfall treatment did not have any effects on the N stocks nor did it alter the effect of compost on N stock at all depths. Cumulative N stock from 0 to 30 cm was similar across OA and rain treatments. Mineral N content (NH_4^+ and NO_3^-) was different only between rain treatments (Fig. 4.2; $F_{2,22} = 11.4$, $p < 0.001$ for NH_4^+; $F_{2,22} = 53.9$, $p < 0.001$ for NO_3^-). Soil NH_4^+ and NO_3^- were higher in drying and rewetting treatment compared to normal rain.

Soil P stocks were altered with compost amendment at only 0 to 5 cm (Fig. 4.1; $F_{1,22} = 6.1$, $p = 0.021$), where compost amended soils had higher total P. Rainfall treatment did not have any effects on the P stocks nor did it alter the effect of compost on P stock at all depths. Cumulative P stock from 0 to 30 cm was similar across OA and rain treatments. Plant available (Colwell) P was only different between amended and unamended treatments (Fig. 4.2; $F_{1,22} = 6.4$, $p < 0.019$). Compost amended soils had higher Colwell-P compared to unamended soils.

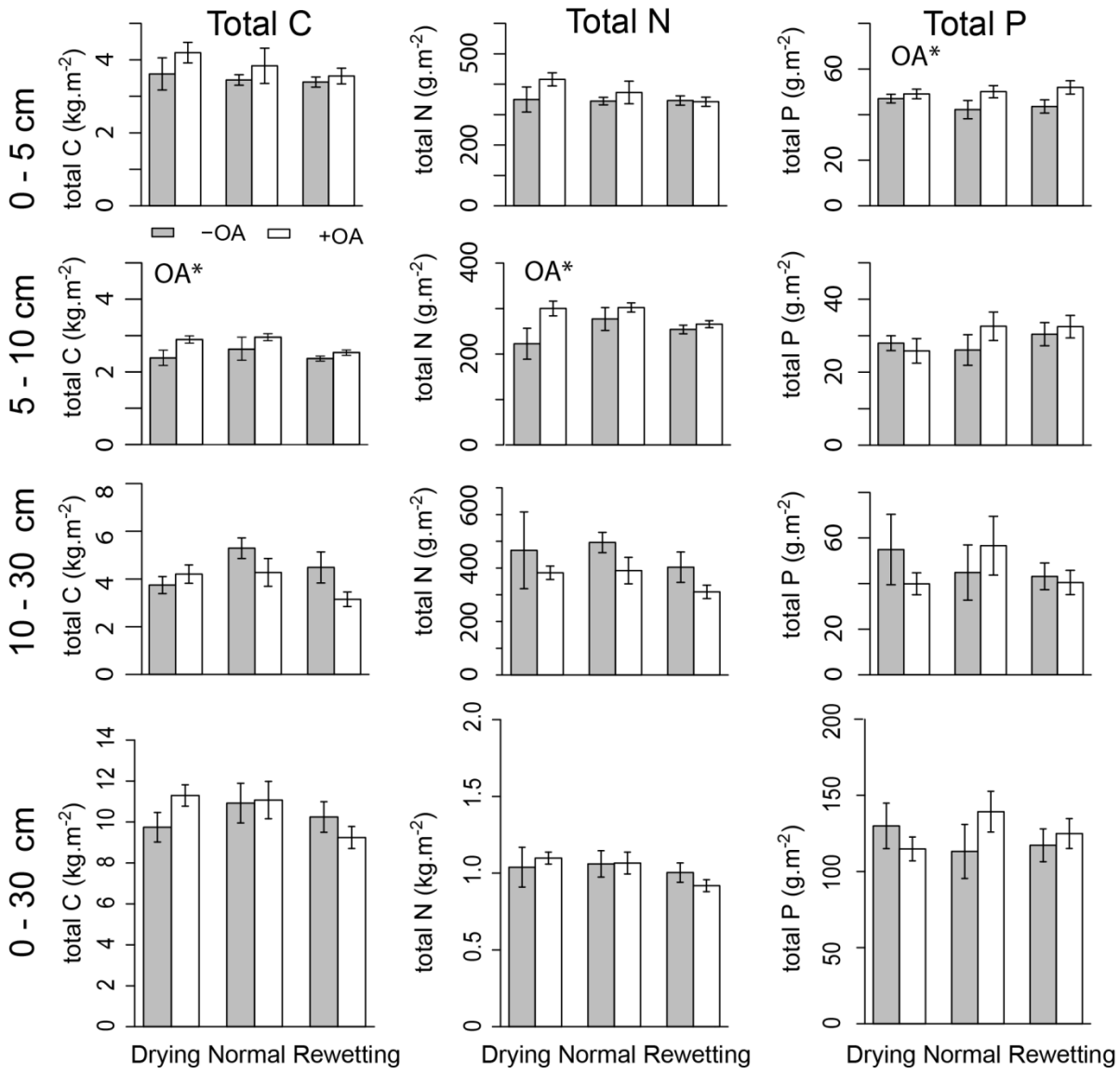


Fig. 4.1 Effects of OA on total C, N and P at 0-5 cm, 5-10 cm, 10-30 cm and 0-30 cm under drying, normal and rewetting treatments. Significant levels are *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (refer in-text for test statistics). –OA = unamended soils; +OA = amended soils.

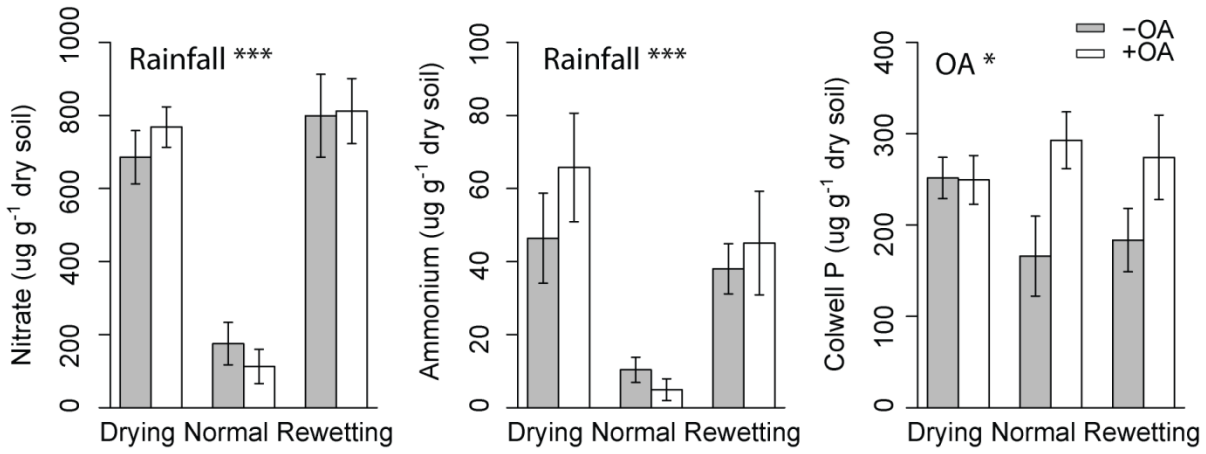


Fig. 4.2 Available nutrients (NO_3^-, NH_4^+ and Colwell-P) for 0 to 5 cm. Significant levels are *p < 0.05, ** p < 0.01, *** p < 0.001 (refer in-text for test statistics). –OA = unamended soils; +OA = amended soils.

4.4. Discussion and conclusion

Prior studies have found evidence that a single application of organic amendment can improve grassland soil organic matter for 14 years after application (Ippolito et al., 2010). In a single surface application of compost to grassland soils, Ryals et al. (2014) found the organic amendment was incorporated into labile and physically protected pools in the soil, with corresponding increase in soil C and N stores. We found that compost improved C and N stocks at 5 to 10 cm and P stocks at 0 to 5 cm regardless of the rainfall regime, but C, N and P stocks at 10 to 30 cm were unaffected by rainfall or compost amendment. Therefore, a single application of compost can benefit C and N stores in this grassland regardless of the rainfall regime.

The longevity of the increase in soil C, N and P will depend on microbial transformation of the organic amendment. In sandy soil, recalcitrance of the amendment is important to avoid rapid decomposition of the organic amendment by soil microbes (Nicolás et al., 2012). In our grassland with sandy soil, we have found that the compost have improved

C, N and P contents in the upper 10 cm. In fact, all the added C, N and P have been retained within the 0 to 30 cm depth during this 3 month experiment (data not shown). A longer time-scale study will be necessary to tell if given sufficient time, these added C, N and P will be sustained in the ecosystem.

We expected rainfall to alter the effects of the compost amendment on grassland plant and below-ground community and thus, alter the total C, N and P contents of the soil. Carbon cycling in annual grassland has been found to be sensitive to changes in rainfall timing as soil respiration and net primary productivity increases with a longer or later wet season (Chou et al., 2008). In a study on Californian grassland, increases in rainfall had little effect on net primary productivity due to relative changes in above-ground and below-ground allocation in plant biomass while nitrate deposition strongly increased net primary productivity (Dukes et al., 2005). However, they found little interaction effect between rainfall and nitrogen deposition. Prolonged drying severely affects soil moisture and microbial activity. Drying has been found to inhibit C and N mineralisation by microbial activity and affects nutrient availability in both manure compost amended and unamended soils (Hueso et al., 2012). In a native prairie ecosystem in Kansas, USA, microbial activity and biomass during variable soil water conditions indicated that drier conditions may have negative feedback on soil C loss but 14 years of rainfall reduction did not change the total soil C pool (Zeglin et al., 2013). Therefore, a complex interplay of above-ground and below-ground is likely to be operating at different timescales, producing the results we have observed.

Although the total stocks did not change with rainfall, it is important to note that available nutrients were different across the rain treatments; as we observed higher NH_4^+ in drying and rewetting treatment compared to normal rain and NO_3^- was highest in the rewetting treatment, followed by the drying and normal rain treatments at 0 to 5 cm. But similarly to results of the total stocks, there was no interaction between the rainfall and

compost treatments. Given primary productivity depends on available nutrients, changes in this pool with rainfall is important.

The observed P accumulation at the 0 to 5 cm with addition of compost indicated the unavailability of P in this soil. When three different organic amendments were added separately to soil, there were increases in organic P pools but available P decreased over time (Malik et al., 2013). Inorganic P rapidly becomes unavailable due to leaching, erosion and association with minerals (Condron and Tiessen, 2005). As our grassland soil has relatively high contents of calcium, aluminium and iron (data not shown), it is likely that upon compost addition, the P has been rapidly rendered unavailable.

When C, N and P were considered from bulk soil at 0 to 30 cm, subtle but important changes that occur in C, N and P contents at different depths were lost. It has been highlighted that the depth of sampling is critical for accurate measurements of changes in soil carbon stocks in response to land management practices (VandenBygaart et al., 2011). For example, when VandenBygaart et al. (2011) compared effects of till and no-till on soil carbon stock, differences were detectable for 0 to 30 cm but not 0 to 15 cm, while the effects of perennial forage were detectable at all sampling depths to 60 cm.

The use of green waste compost as soil amendment provides a genuine possibility to increase soil C for carbon sequestration as this type of compost are otherwise disposed to landfill (Powlson et al., 2012). While compost amendment can increase soil C, N and P stocks in an intensively grazed grassland irrespective of rainfall conditions, these subtle improvements were very much soil depth-dependant.

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Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Setup study design, lab work, analysed data and wrote the manuscript.	90

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Antonio Patti	Involved in study design, writing manuscript	2
Timothy Cavagnaro	Involved in study design, writing manuscript	2
Michael Rose	Enzyme activity analysis, writing manuscript	4
Roy Jackson	Involved in study design, writing manuscript	2

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Chapter 5: Low diffusivity of soil enzymes: An evolutionary and functional perspective

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Abstract

Ecoenzymes are important mediators of degradation, transformation and mineralisation of soil organic matter. Their activities rely on their encounter with substrate either by diffusion of substrate or enzyme. However, little is known about the relative contribution of free and bound ecoenzymes to decomposition of soil organic matter (SOM), particularly for oxidases. For the soil microbes, there is relatively little payback on its investment in the production of these energetically expensive enzymes if disproportionate amount of them are lost by diffusion or to cheaters. We measured the activity of two oxidases (phenol oxidase and peroxidase) and two hydrolases (β-glucosidase and acid phosphatase) in three contrasting soils. Most of the total enzyme activities for all four ecoenzymes were associated with bound rather than free fractions. This suggests low diffusivity of ecoenzymes. Low diffusivity of oxidases may particularly constraint SOM turnover. Additionally, low diffusivity of ecoenzymes in the soil environment maintains benefits to the enzyme producer and may explain the evolutionary stability of ecoenzymes.

Keywords: extracellular enzyme; lignocelluloses; microbial activity; nutrient cycling; carbon cycling

5.1 Introduction

Soil microbial communities play a major role in regulating the cycling and flow of energy and nutrients in the environment (Conrad, 1996; De Deyn et al., 2008; Van Der Heijden et al., 2008). Advances in the field of molecular ecology have enabled ecologists to more fully explore the diversity of organisms that reside in soil, and relate changes in soil diversity to those occurring above-ground (Wagg et al., 2011; Wardle et al., 2004). Such information, when combined with measures of the functioning of soil biota, allows us to understand the mechanisms underlying soil-mediated energy and nutrient cycling.

Ecoenzymes are enzymes not contained by membranes of living cells (Sinsabaugh et al., 2009). Given their key role in the cycling of nutrients and carbon in the soil, they are increasingly recognized as important in global climate regulation. Lignocellulose-degrading ecoenzymes play an important role in the decomposition of plant-derived organic matter, and thence, the sequestration of carbon in the soil. For example, hydrolases, such as cellulase are important in cellulose degradation (Shi, 2010), and oxidases such as phenol oxidase and peroxidases, are involved in lignin degradation, humification, carbon mineralisation and dissolved organic carbon export (Sinsabaugh, 2010).

The encounter of an ecoenzyme─with its substrate, and ultimately microbial decomposition of organic matter, relies largely on the diffusion of the enzyme away from its parent cell, and/or the diffusivity of its substrate in the soil (Burns et al., 2013; Koch, 1990). Emerging evidence suggests that ecoenzyme producers do not operate in isolation. For

example, through mechanisms such as quorum sensing, they interact with other microbial degraders, thereby ensuring that they receive a positive return on their investment in enzyme production (Burns et al. 2013). Nevertheless, diffusion losses and cheating are still problems faced by ecoenzyme producers (Allison, 2005; Allison et al., 2011).

Soil microbes may partly circumvent these problems if their ecoenzymes are quickly sorbed onto soil constituents (e.g. clay, humic colloids, cell debris) in close proximity to the producer. To this end, Allison and colleagues (2005; 2011) have hypothesised that selection will operate in favour of producers of ecoenzymes with low diffusivity. We contend that studies on free and bound ecoenzymes may help explain if or why this cooperative mechanism is evolutionarily stable in soil microbial communities.

Interestingly, there is little work on the relative size of free and bound fractions of ecoenzymes in soils (Štursová and Baldrian, 2011). The few studies of the free fraction of ecoenzyme activities have focused on hydrolases (Marx et al., 2005; Stemmer et al., 1998; Vepsäläinen, 2001), neglecting oxidases, which play important roles in lignin-degradation. With increasing interest in the processes governing the cycling and fate of carbon in soils, this represents an important knowledge gap. Consequently, we present here results of a study in which we compared the levels and patterns of ecoenzyme activity in the bound and free fractions of enzymes for two oxidases (phenol oxidase and peroxidase) and two hydrolases (β-glucosidase and acid phosphatase) in three contrasting soils. Results are considered in the context of the implications on soil elemental cycling and evolution of ecoenzymes.

5.2 Materials and Methods

We compared enzyme activities in three contrasting soils (table 5.1). For each soil, 50 g of soil was wet to 20 % gravimetric soil moisture, and incubated for three days at room temperature. Following incubation, five 1 g samples were taken and each shaken in 50 mL of sterile reverse osmosis (RO) water for an hour to obtain homogeneous soil slurries. Soil enzyme activities were then measured following two different methods of sample preparation to obtain (i) total activity of bound and free fractions of enzyme, and (ii) activity of the free fraction of enzymes (Fig. 5.1). First, total enzyme activities were determined on three 0.5 mL analytical replicates taken from each homogenised soil slurry. Second, the homogenised slurry was centrifuged at 1,900 g, and free enzyme activities were determined on six 100 uL analytical replicates.

Table 5.1. Soil characteristics at study sites

	Werribee clayey soil	Cranbourne sandy soil	Stony Creek loamy soil
Location (Lat, Long)	37°53'S, 144°40' E	38°11'S 149°19'E	38°35'S, 146°03'E
Landuse	Horticultural farm	Horticultural farm	Pastureland
Classification	Red sodosol	Podosol	Brown dermosol
pH (H_2O)	7.79	7.79	5.30
C: N ratio	9.1	13.0	13.7
Organic matter (%)	3.9	1.3	11.3
Total N (%)	0.20	0.12	0.47
Water holding capacity (g H_2O g^{-1} dry soil)	49.5	20.8	55.3

Enzyme activities were measured as follows: β-glucosidase (BGL), phosphatase (PHOS) and phenol oxidase (PPO) activities were determined with procedures modified from Allison and Jastrow (2006) using the substrates 5 mM *p*-nitrophenyl-β-D-glucopyranoside, 5 mM *p*-nitrophenyl-phosphate and 50 mM pyrogallol/ 50 mM EDTA respectively. Peroxidase (POX) was determined with a procedure modified from Frey *et al.* (2000) and Johnsen and Jacobsen (2008) using the substrate 1 mM 3,3',5,5'- tetramethylbenzidine. Substrate solutions were made up using 100 mM, pH 7, 4-morpholinepropanesulfonic acid buffer (except the peroxidase assay which used 100 mM, pH 5, acetate buffer).

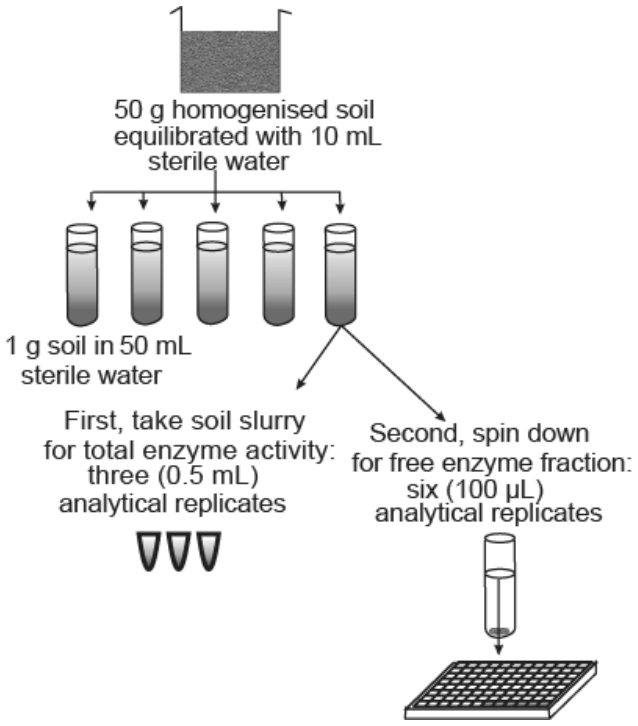

Fig. 5.1 Sample preparation prior to enzyme assays to obtain total and free enzyme activities.

For the total enzyme activity, 0.5 mL of soil slurry was combined with 0.5 mL of substrate solution. For the free fraction of enzymes, 100 μL of substrate solution was added to 100 μL of soil extract on a 96-well microplate. The sample-substrate mix was incubated for 10 mins (peroxidase), 1 hr (phenol oxidase) and 2 hr (β-glucosidase, phosphatase), followed by colorimetric measurement of reaction products. Background soil controls containing soil slurry or extract with sterile RO water and substrate controls containing substrate and buffer were prepared for all enzymes. Absorbance was measured at 405 nm except for peroxidase which was measured at 450 nm. The activity of bound enzyme fraction was calculated as:

Bound enzyme activity = Total enzyme activity - free enzyme activity (Eq. 1)

The variations between the three soils and enzyme fractions were compared using a split-plot ANOVA. The soils were treated as the main between block treatment factor in the model, and enzyme fraction as the main within block treatment factor. The main effect differences in enzyme activity between soils for each enzyme fraction were compared using Tukey HSD. We also calculated ecoenzyme diffusibility as:

Ecoenzyme diffusivity = (Free enzyme activity)/(Total enzyme activity) × 100 % (Eq. 2)

The differences in ecoenzyme diffusivity among the three soils were compared using one-way ANOVA followed by post-hoc multiple comparisons using Tukey HSD. All statistical analysis was performed using R2.14.1 (R Core Team 2012).

5.3 Results

The activities of the bound fraction of the four ecoenzymes were significantly higher than those of the free fraction, in all three soils (Fig. 5.2, Table 5.2). The differences in

patterns of enzyme activity between the free and bound ecoenzymes across soils revealed significant interaction between the soil and enzymes fractions. For example, whereas the bound PPO activity was higher in the Stony Creek soil than the Werribee and Cranbourne soils, free PPO activity was lower in Stony Creek soil than Werribee and Cranbourne soils. Similarly, while there were clear differences between bound POX activities in the soils, the same was not true with the free fraction of enzymes. Such inconsistencies between bound and free enzyme activities were observed for all the enzymes except PHOS.

Table 5.2. Split-plot ANOVA of enzyme activity variations due to soil and assay method employed; F-values shown and p-values indicated as asterisks

	PPO activity	BGL activity	POX activity	PHOS activity
Error: Block				
Soil	**161.7 *** **	**34.08*** **	**42.71 *** **	**1580 *** **
Error: Within				
Enzyme fraction	**3704 *** **	**99.59 *** **	**540.38 *** **	**2148 *** **
Soil: Enzyme fraction	**200.7 *** **	**43.68 *** **	**24.22 *** **	**1545 *** **

#: PPO = phenol oxidase, BGL = β-glucosidase, POX = peroxidase, PHOS = acid phosphatase. β-glucosidase activity was very low in Cranbourne soil and therefore excluded from comparison. p-value significance code ***, **, * indicates significance of <0.001, <0.01, <0.05 respectively.

The diffusivity of the ecoenzymes measured here differed between the soils, and these difference were not consistent among the ecoenzymes. For example, in Werribee soil, the free enzyme activity (Table 5.3) ranged from 3.9 % to 14.7 % of total enzyme activity; in the Stony Creek soil, the free enzyme activity represented less than 10 % of total enzyme

activity; and in the Cranbourne soil, the free enzyme activity ranged from 12.8 % to 17.2 % of total enzyme activity. Furthermore, we found no consistent patterns for the relative diffusivity of oxidases or hydrolases. For example, in the Werribee soil, whereas the diffusivity of the PPO and BGL were high, that of the PHOS was low and the POX intermediate. In contrast, in the Stony Creek soil, the diffusivity of the POX was higher than that of the other three enzymes.

Table 5.3. Enzyme diffusivity as a percentage of that enzyme's total activity (mean ± SD) in Werribee, Stony Creek and Cranbourne soils.

Soil	PPO diffusivity	BGL diffusivity	POX diffusivity	PHOS diffusivity
Werribee	14.7 ± 2.26 ^{a}	13.23 ± 3.52 ^{ab}	8.72 ± 1.01 ^{bc}	3.88 ± 0.65 ^{c}
Stony Creek	3.66 ± 0.40 ^{a}	1.93 ± 1.29 ^{a}	8.72 ± 1.34 ^{b}	1.72 ± 0.08 ^{a}
Cranbourne	12.8 ± 0.77 ^{a}	NA$^{\#}$	17.2 ± 3.11 ^{a}	13.2 ± 1.58 ^{a}

#: β-glucosidase activity was very low in Cranbourne soil and therefore excluded in comparison of diffusivity. Different superscript letters refer to significant differences ($p < 0.01$) in enzyme diffusivity in each soil based on one-way ANOVA followed by post-hoc comparison using Tukey HSD. PPO = phenol oxidase, BGL = β-glucosidase, POX = peroxidase, PHOS = acid phosphatase

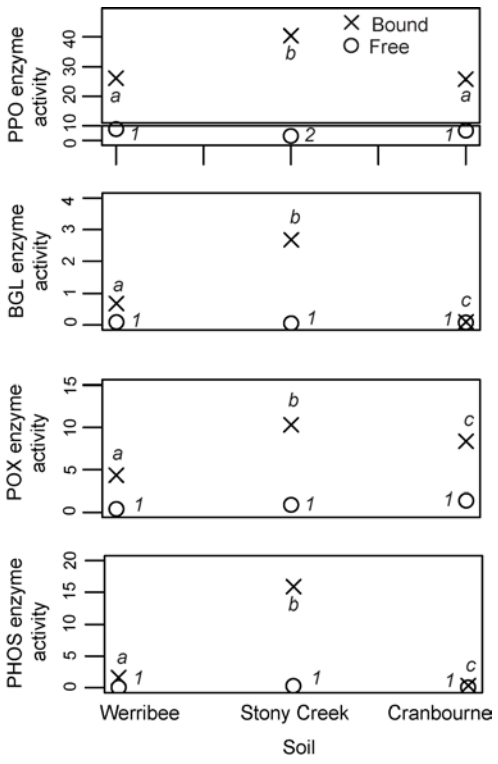

Fig. 5.2 Bound and free enzyme activities in the three soils. Enzyme activity is calculated as μmols pNP g^{-1} dry mass h^{-1} for PHOS and BGL, μmols TMB g^{-1} dry mass h^{-1} for POX and μmols pyrogallol g^{-1} dry mass h^{-1} for PPO. The results represent mean ± standard deviation. PPO = phenol oxidase, BGL = β-glucosidase, POX = peroxidase, PHOS = acid phosphatase. Different letters (for comparison of bound enzymes) and numbers (for comparison of free enzymes) refer to significant differences ($p < 0.05$) in enzyme activity in each soil based on post-hoc comparison of main effects using Tukey HSD. Note that the standard deviation is not shown here (see supplementary data for mean±standard deviation).

5.4 Discussion

Most of the total activity of the four ecoenzymes measured was associated with the bound, rather than the free, enzyme fraction. This indicates that the diffusivity of these enzymes in the soil was low. This finding is consistent with earlier studies of soil hydrolases (Kandeler, 1990; Štursová and Baldrian, 2011; Vepsäläinen, 2001). Our data showed that the diffusivity of the oxidases were no more or less than the hydrolases. This is, to our knowledge, the first study to examine the diffusivity of oxidases in soil. Previous studies have focused primarily on the diffusivity of hydrolases (Marx et al., 2005; Masciandaro et al., 2008; Stemmer et al., 1998; Vepsäläinen, 2001). The activity of oxidases has important implications for sequestration of carbon. They depolymerise complex organic matter, thereby allowing hydrolases to mineralise otherwise inaccessible organic matter yet at the same time

they are involved in humification processes to form potentially more stable carbon forms. Low diffusivity of oxidases may therefore limit organic matter breakdown and reduce humification. This suggests that certain components of soil organic matter can be broken down and/or transformed, subject to oxidases gaining access to them.

Many edaphic factors, such as particle size distribution, mineralogy, organic matter content and soil structure will influence the diffusion of soil enzymes (Allison and Jastrow, 2006; Marx et al., 2005; Quiquampoix and Mousain, 2005). Since the free enzyme pool relies upon the availability and flow of water in the soil for movement, changes in soil moisture could influence the likelihood of an enzyme encountering a suitable substrate (Ekschmitt et al., 2005), which in turn affects organic matter decomposition and/or stabilisation. This may be particularly important in the light of current projections of a drying climate in many regions of the world (Meehl et al., 2007).

Interestingly, the relatively low diffusivity of oxidases in our soils is in contrast to studies of their diffusivity in litter layers, where they are more mobile than hydrolases (Valášková and Baldrian, 2006). While the reasons for these differences remain unknown, this difference makes sense if we consider that fungi dominate oxidase secretion in the litter layer. Ecoenzyme production and secretion are energetically expensive. Selection to low enzyme diffusivity has been proposed as viable strategy for microbes to manufacture and secret ecoenzymes in the presence of diffusion losses and free-riding microbes that do not secret ecoenzymes (Allison, 2005; Allison et al., 2011). When investments are only shared locally, the enzyme producer minimises its losses (Sherratt et al., 2009). Equally, the enzyme producer may benefit from the enzymatic activity of other producers within the local group (Ekschmitt et al., 2005). In the litter layer, oxidases may diffuse further but the gains from the enzyme activity may be still appropriated by the enzyme producers through the hyphal network.

In this experiment we compared three contrasting soils and four enzymes. While we caution that it will be necessary to examine a greater number of soils and enzymes before broad generalizations can be made, we do note that the differences in diffusivity among treatments in this experiment were more strongly influenced by the soil. It was also observed that inconsistent differences in patterns of enzyme activity between the free and bound ecoenzymes. This has important methodological implications. For example, PPO activity was highest and lowest in Stony Creek soil in the bound and free fractions respectively. Given that PPO activity is related to the mineralisation of lignin and other secondary compounds, the results from the two ecoenzyme fractions could lead to different interpretations of the carbon mineralisation rates in different soils. Consequently, interpretation of the ecological significance of soil enzyme data should take into account the differences in enzyme fractions. This is important not only when comparing among studies, but also in interpreting soil enzyme activity data more generally.

Taken together, our results demonstrate that the diffusivity of oxidases appears to be no more or less in the soils tested here than for hydrolases. This low diffusivity can be a constraint to decomposition of soil organic matter. Furthermore, the relatively low diffusivity of ecoenzymes in the soil environment may help ensure that the benefit of energetically costly ecoenzyme production is received by the enzyme producer, and this economic principle helps to explain the evolutionary stability of ecoenzymes.

5.5. Acknowledgements

This work was supported by funding from Monash Sustainability Institute Australia, and Brown Coal Innovation Australia. TRC also thanks the ARC for the award of a Future Fellowship (FT120100463).

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Chapter 6: What can ecosystem approaches do for soil management?

Abstract

Soil is central to human wellbeing through its provision of critical ecosystem services including food and clean water. These soil ecosystem services emerge through the self-organising nature of the soil system. This short essay highlights the benefits of an ecosystem approach to soil management from the application of the concept of ecosystem health, as translatable to soil health, and ecosystem theories relevant for the management of soil for human wellbeing. In this essay, I argue that there is still room for expansion of the application of the soil health concept, and that the use ecosystem theories will allow us to better understanding the complex, soil system.

6.1 Introduction

Soil is central to human wellbeing in its provision of food, fibre, fuel, clean water and regulation of greenhouse gases. In this changing world, securing those benefits from soil will be the key to human living on this land (Janzen et al., 2011). These benefits from the soil are emergent properties of the soil, arising not only from the sum of its component but as a result of the interaction among the components. It is the emergent properties of the soil system that reveal studying complex soil system requires an trans-disciplinary approach to enable us to weave disparate threads of information on soil system behaviour into meaningful picture of the nature of soil, of which meaning is derived mainly at human-relevant scales. This short essay is partly inspired by Wolfgang von Goethe, an ambidextrous scientist, writer and politician, who was quoted to have said "*If we take a man as he is, we make him worse; but if*

we take him as he should be, we help him become what he can be". In this essay, I deliberate over the usefulness of ecosystem health and ecosystem theories to understand soil's nature and therefore bridge our grand needs to its diminutive inhabitants and their immense landscapes. It does not seek to provide a comprehensive review of current states, but provide short summaries, and hopefully entice the readers to further explore this realm of possibilities that may benefit soil management.

6.2 What can ecosystem health do for soil management?

"If we take a man as he is, we make him worse; but if we take him as he should be, we help him become what he can be" Wolfgang von Goethe

If we take soil as it is, we make it worse; but if we take it as it should be, we help it become what it can be. The soil is a living system. With soil health, it is possible to consider degraded system as transiently sick and the system can regain immunity when it regains health. The main limitation is likely to be the question of how sick – will it spiral down to death or will it regain immunity and be revived given the right medicine, in other words, the right management?

Taking a cue from the concept of holistic health, health is not simply the absence of disease but encompasses wellbeing (Waltner-Toews, 1996). A healthy soil system should be judged not only by its ability to supply ecosystem services, but also its resilience (which includes robustness, adaptability and flexibility), self-reliance and empowerment (see Gallopin, 1995 for his detailed interpretation of these attributes). These attributes are briefly explained here. A healthy soil will be judged by its economic performance, but this is not all. The collective attributes of a given soil banded under resilience refers to the ability to

respond to and maintain a certain level of integrity under the ever changing environment. A healthy soil is self-reliant, that is it responds dynamically to external changes but it ultimately determines its own trajectory. And finally, empowerment refers to the soil system's capacity to influence the external world. This implies that the system may generate changes in other systems in order to fulfill its goals.

These wellbeing attributes take into account not only the present but the potential for the future. As such, this approach provides possibility for fitting soil management into economic valuation-based ecosystem, biodiversity and ecosystem services management. In doing so, this concept of soil health presents us with a meaningful way to describe soil in its complex gestalt system form while providing a linkage for translating science into practice. As a system with multiple stakeholders, this form of meaningful analogy will aid communication and problem-solving as people can identify with the concept of health.

From an intellectual viewpoint, it sets the stage for a much needed "intellectual smoothie", for health considers in its entirety biochemical and physical states of an organism or ecosystem. Coincidentally, Wolfgang von Goethe, who inspired this essay, is a very good example of cross-fertilisation in science, poetry, drama and philosophy. Cross-fertilisation can, has and will continue to benefit soil science. For decades, soil science has adopted ideas, approaches and methods used in medical research. An example that comes easily to mind is the application of molecular technique that has led to insight of the overwhelming diversity of soil microorganism and increasingly, what they do. The tremendous progress in the study the brain and mind is a mark of an era of possibilities. Once considered a black box, advances are slowly beginning to unveil the workings of this complex network system. This suggests that complex systems can be understood and there is much we can learn from such studies of complex systems. With better understanding of the soil system, the gibberish noise and chaos as revealed by the current state of knowledge of the inner workings of soil system will turn

into ordered, meaningful patterns. These patterns, in fact, are starting to unfold with methodological advances in molecular approaches.

6.3 What can ecosystem theories do for soil management?

The fundamental concern of management is to preserve ecological integrity, such that the soil system continues to meet human demands, therefore, an important question is what can ecosystem theories do for the management of soil? Which theories are relevant for the comprehension of the complexity of soil? Clearly, other authors have been considering the application of ecosystem theories for soils, including Iain Young and John Crawford's (Crawford et al., 2012; Young and Crawford, 2004) introduction to self-organising nature of soil system and Dirk van Apeldoorn and his colleagues (2011) who explored the application of ecosystem theories for farm management. Young, Crawford and colleagues proposed that important abiotic - biotic interactions result in the self-organisation of the soil system and we need an integrated approach to studying soil. Van Apeldoorn and his colleagues used the existing key heuristics of resilience, in which they included panarchy, to examine a dairy agroecosystem and propose their consequences for managing agroecosystems. But the extent of the application of ecosystem theories to soil studies is still at its infancy for various reasons, including the difficulty of turning complex theoretical jargons into an applied science. Nevertheless, such attempts should be made, even if it entails great risks, for its potential is there for great rewards to those who succeed in marrying the ecosystem theories to the management of soil.

As the soil system is dynamic, it evolves with prevailing conditions while leaning and learning from its memory or historical legacy (Keiser et al., 2011; Strickland et al., 2009). Additionally, humans are a significant component of this unique system and this component

actively provides regulatory inputs. These suggest that to understand soil, there is a need for a theoretical framework – one that "allows us to arrange the world in a way that gives new insights, to ask new questions, and to see further" (Warren, 2005).

BOX 1

The hierarchy theory provides clues to interactions between processes and components at seemingly different space and time scales. The specifications of hierarchy theory are not detailed here as Warren (2005) and Holling (2001) have carefully teased apart the meaningful bits but in a nutshell: each dynamic level (within the horizontal or vertical structure of the conceptual model) is semi-autonomous; the freedom exists within the upper level constraint. The persistence of vital communication between the levels, i.e. information transferred from bottom (faster and smaller level) up (slower and coarser level) sustains the overall system.

This theoretical framework employs hierarchy theory (see box 1) to elucidate the importance of cross-scale interactions in soil ecosystem (fig 6.1A). At each scale or level, dynamism is captured by the adaptive cycle and the interaction between these adaptive cycles gives rise to panarchy (Gunderson and Holling, 2002) (fig 6.1B). In a nutshell, panarchy is a nested set of adaptive cycles that communicate, self organise and determines the integrity of a soil ecosystem. For example, soil processes at farm scale contributes to ecosystem functioning at watershed scale which leads to ecosystem services benefits regionally. There are also within-scale interactions among the edaphic-ecological-human-meteorological dimensions. These interactions within and across scales determine the emergent properties of the soil system.

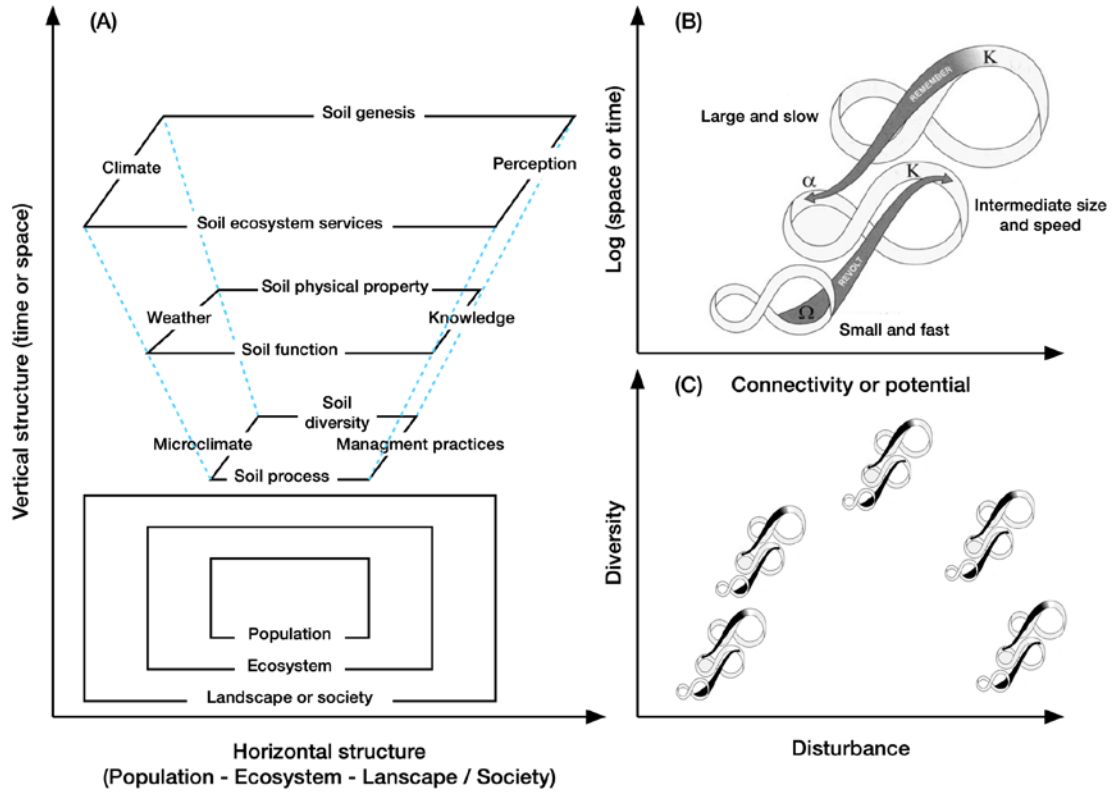

Fig 6.1. Conceptual model: Multidimensional hierarchy of soil ecosystem (alternative socioecological system). (A) Horizontal structure encompasses various interacting groups. The multidimensional vertical structure encompasses edaphic-ecological-human-meteorological dimensions interacting over time and/or space scales. The model focuses on processes. (B) Panarchy combines hierarchy theory with the adaptive cycle (figure b from Gunderson and Holling 2001). The adaptive cycle is characterised by continuous phases of growth (r) – accumulation (K) – release (Ω) – restructuring (α). Two types of connections are emphasized, i.e. revolt and remember; the former referring to the cascade-up effect while the latter refers constraints from above. (C). Modified panarchy model combines hierarchy theory with intermediate disturbance theory. The intermediate disturbance hypothesis suggests that moderate disturbance benefits diversity (Grime 1973), in this case, supporting soil functional diversity and resilience. Disturbance at a level may or may not be experienced across levels as a result of connectivity.

It has been proposed that the self-organizing nature of the soil system is controlled by a set of critical processes - carbon transformation, nutrient cycling, maintenance of soil structure, and biological regulation of soil population (Kibblewhite et al., 2008).

Conservation of the communication between these key processes is proposed as the key to maintaining the flow of ecosystem services. A important fraction of these communications will occur through the soil living components. If indeed the conservation of communication between these key processes underlies a healthy soil system, an inquiry process focusing on flows or activity rather than seeking to set clear boundary of components of the system may be most beneficial. As such, we may better understand the relationship between these key processes and the implications of changes in their relationship as a result of human activity by employing a soft-system methodology. This approach to the subjective moves us from focus from modeling components towards modeling relationships (see review by Checkland, 2000); from who is there towards how relationships between soil living components translate into soil functions, and thus soil health.

And finally, a merger between panarchy and the intermediate disturbance hypothesis may also be useful. Here, the product of this merger is called the modified panarchy model (fig 6.1C). The trajectory of the soil system is determined by the impact and responses of the aforementioned key processes to disturbances. The result is a soil system that is "both creative and conservative through the dynamic balance between rapid change and memory, and between disturbance and diversity and their cross-scale interplay" (Folke, 2006). At moderate levels of disturbance to the soil system, the system self organizes to maximize diversity. This diversity refers to the dimension at consideration; for example in the ecological dimension, it may refer to biodiversity while in the human dimension it may refer to diversity in management responses.

From this framework, it becomes clear that knowing higher constraints improves predictability yet at the same time exposes uncertainty, as we have observed in the case of global change. It provides us with a mean to identify the space-time scale of relevance to decision making in the human sphere.

6.4 Conclusion

The soil system has potential to be more than what it is currently. Human have created maladaptive adaptive cycles in our attempts to assist soil to perform. If the self-organizing nature of soil ecosystem is combined with the unique capacity of human-laced systems for intention and foresight, technology and communication (Holling 2001), recovery is possible.

The central argument of this essay is that the management of soil health can benefit from an ecosystem approach to its management. In particular, understanding the complexity of soil system using ecosystem theories will allow us to better manage soil for human wellbeing. This theoretical framework highlights the interaction between space-time scales. Therefore, future research would benefit from incorporating scaling, combining scales meaningful to soil inhabitants and larger spatial scales realistic to human management (farm-scale and landscapes); and from short-term to long-term monitoring of soil ecosystem. These have only been weakly addressed thus far. If we can remedy our approach to soil, soils can remedy their states and satisfy our many demands on it.

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Epilogue: concluding remarks

"Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning" Winston Churchill

In this concluding chapter, I intend not to reiterate the findings of the preceding chapters, which have been nicely done by the conclusion section of each chapter and the thesis summary. Instead, I dare briefly venture to cover the broader implications of the studies that I have carried out, what I have learnt of and from soil ecosystems, and where do we go from here.

The studies performed in this thesis are generally of short-term or theoretical nature. If one considers the nested hierarchical nature of an ecosystem as a set of cogged wheels, these short-term dynamics represent parts that keep the machinery running. They are therefore important for the functioning of the system and ultimately determine what remains in soil. A key player in the soil ecosystems are microbes. Microbes are endowed with metabolic flexibility, physiological tolerance and rapid evolutionary adaption (see Allison and Martiny, 2008; Griffiths and Philippot, 2013 and references therein). As a result, what is merely weeks and months to humans could have been many generations for microbes[1]. Nevertheless, it is necessary to remember that short-term dynamics does not inform long-term stability, and therefore, must be complemented by long-term studies.

Prudent management of soil resources in the face of interacting global drivers of change require knowledge of plant and soil biota responses to environmental change. While

[1] On a side note, these unique characteristics of microbes makes soil ecosystem a gift for researchers to use as a model to understand the effects of global change within the time scale of a human life time.

plant responses have been reasonably studied, less is known of belowground responses. The use of terrestrial model ecosystem approach enables us to examine biological complexity while allowing some control over environmental variability (Jones et al., 1998; Kandeler et al., 1998; Moore et al., 1996; Morgan and Knacker, 1994). Studies on global change are complex in essence and the manipulation of multiple global change stressors in the field are costly, time-consuming and confounded by other environmental change. As such, the use of such model ecosystems allow us to first identify underlying ecological principles that can then be further confirmed with field studies. Based on the results from the terrestrial model ecosystem, soil microbial community from this grassland are resistant and resilient to drying but the microbial activity, and therefore, soil functions are not resistant to drying. This suggest that the duration of drying will have possible important implications on biogeochemical cycling.

A common contention in the study of the soil ecosystem is its complexity. The results of the experiments here indicate that within the chaos are patterns that are comprehensible and perhaps even predictable. In social science, the term path dependence is used to describe the concept that history matters. In this view, while the emergent property of the soil may seem too complex, it is still constrained in its response to a certain extent due to the memory retained by soil. Studies have shown the importance of historical legacy in determining the response of soil ecosystem to further disturbance (Griffiths and Philippot, 2013; Keiser et al., 2011; Martiny et al., 2006; Pagaling et al., 2013). Based on these studies, similarity between the new and past disturbances partly determines the ability of the soil ecosystem to respond to new disturbances.

On a different note, a reader of this thesis may question the lack of quantitative expression of each soil function, as would have been most meaningful from an agronomic point of view. For example, it would be most useful in agronomic terms to see the following

statements: *Among the organic amendments tested (i.e. fresh green waste, compost and biochar), the application of compost benefits both soils most over the time scale examined. The benefits include improvements in stock and available nutrients.* This is by no means short-sightedness on the author's behalf and it must be emphasised that such quantitative expressions are no less important, but the focus of the thesis is patterns – patterns of soil ecosystem that represent meaningful abstraction of the system, and patterns that explain the underlying mechanisms resulting in those very quantitative expressions of soil functions that are important to agricultural management. I hope that we can then apply a combination of this comprehension of the nature of soil and some tender nurturing to improve overall soil health for the benefit of human wellbeing.

Where do we go from here? As with most studies, the experiments here answered some questions and raised more questions. For one, what are the relative influences of the chemical nature of the carbon and biological mechanisms on soil organic matter transformation? And as such, do some soils have greater capacity to respond than others? Secondly, organic input altered the soil microbial community structure and composition. Have we permanently altered the trajectory or dynamics of the system, and if so, what are the implications of these changes in the longer term? Thirdly, while ecological and functional stoichiometry showed potential for explaining soil microbial community response to organic input in the incubation experiment, further studies need to examine the temporal and spatial scales at which the patterns observed are effectively transferred across to guide its application. Developments in metagenomics, metatranscriptomics, metaproteomics and molecular chemistry are all promising great excitement to further improve our understanding of the soil ecosystem, which does what in the transformation of soil organic matter and what drives the inhabitants to do more or less or differently. Recent development in meta-analytical approaches is also allowing us to maximise the use of existing data, extracting

information hidden amidst the noise in small datasets which on their own, may not necessary be noticeable.

As is promised at the start of this section, I shall keep it short. I do not attempt to do justice to ideas in the preceding paragraphs. I wish the next person to stumble down the rabbit hole to many great adventures. May their imagination help them in their discovery of the workings of this immense realm existing beneath our humble feet.

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